The regulatory and effector functions of B cells in ANCA-associated vasculitis

Lepse, Nikola

THE REGULATORY AND EFFECTOR FUNCTIONS OF B CELLS IN ANCA-ASSOCIATED VASCULITIS

Nikola Lepse

Groningen 2014

Studies presented in this thesis were financially supported by
Groningen University Institute for Drug Exploration (GUIDE)
Reumafonds
European Union Seventh Framework Programme (FP7/2007-2013)
Jan Kornelis de Cock Stichting

The printing of this thesis was financially supported by

University of Groningen

University Medical Center Groningen

Groningen University Institute for Drug Exploration (GUIDE)

Reumafonds

Hycult Biotech

Cover design
Nikola Lepse & Off Page

Thesis lay-out and printing
Off Page, Amsterdam
www.offpage.nl

ISBN (printed): 978-90-367-7018-7

ISBN (digital): 978-90-367-7017-0

rijksuniversiteit
groningen

THE REGULATORY AND EFFECTOR FUNCTIONS OF B CELLS IN ANCA-ASSOCIATED VASCULITIS

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. E. Sterken
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 18 juni 2014 om 12.45 uur

door

Nikola Lepse

geboren op 15 januari 1987
te Riga, Letland

Promotores
Prof. dr. P. Heeringa
Prof. dr. C.A. Stegeman

Copromotor
Dr. W.H. Abdulahad

Beoordelingscommissie
Prof. A.D. Salama
Prof. dr. R. Goldschmeding
Prof. dr. N.A. Bos

Paranimfen
Niels van der Geest
Nato Teteloshvili

To my loved ones
Maniem mīļajiem

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION AND AIM OF THE THESIS

ANCA-ASSOCIATED VASCULITIS

Vasculitis is inflammation of the blood vessel(s) that damages the vessel wall [1]. It causes pathological changes including, thickening, narrowing and scaring of the vessel wall that can disturb normal blood flow and may result in organ failure. The classification of vasculitides is complex because vasculitis can affect blood vessels of any type and size; it can be limited to a single organ or involve several organ systems. Moreover, vasculitis can occur either as a primary disease, usually of an unknown cause, or it can manifest secondary to another pre-existing condition [1]. A widely accepted classification system for the subdivision of different types of vasculitides has been described in the 2012 International Chapel Hill Consensus Conference on the Nomenclature of Vasculitides [1]. According to this system, primary vasculitides are categorized mainly based on the size of the blood vessels that are predominantly affected, e.g., large vessel or small vessel vasculitides (table 1).

A sub-group of small vessel vasculitides are distinguished from other forms of vasculitis by the presence of circulating anti-neutrophil cytoplasmic antibodies (ANCA). Three disorders with similar clinical and histopathological features are classified as ANCA-associated vasculitides (AAV). These comprise granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA) [1, 2]. The common clinical manifestations of AAV are necrotizing inflammation of small- to medium-sized blood vessels with no or little deposits of immunoglobulins or complement in the vessel wall. AAV can be limited to a single organ, but usually several organ systems are affected with a predilection for the respiratory tract and kidneys [2].

The mortality rate of patients with untreated AAV is very high and reaches 80% at 1 year [3]. In the 1960s therapy with cyclophosphamide and glucocorticoids was introduced, leading to significantly improved prognosis [3]. More than 90% of patients achieve clinical remission with this treatment regimen and it has been the standard therapy for several decades [4]. Nevertheless, long-term treatment with this immunosuppressive regimen has a high risk of severe side-effects, including permanent infertility and the development of malignancies [5, 6]. Thus, less toxic treatment strategies are necessary. A breakthrough in AAV treatment came in 2010, when it was demonstrated that B cell depletion therapy with a chimeric monoclonal anti-CD20 antibody (rituximab) is as effective as standard immunosuppressive therapy for induction of remission in AAV patients [7, 8]. Ongoing studies are investigating if continuous B cell depletion is an effective strategy for remission maintenance [9].

THE ROLE OF ANCA IN VASCULAR INJURY

The first evidence describing the existence of antibodies against neutrophils was published in the early nineteen eighties. In 1982, Davies and colleagues discovered that a factor, which stained the cytoplasm of neutrophils by indirect immunofluorescence, was present in serum of a small group of patients presenting with segmental necrotizing glomerulonephritis [10]. This observation received little attention until

Table 1. Names of vasculitides adopted by the 2012 International Chapel Hill Consensus Conference on the Nomenclature of Vasculitides*

- Large vessel vasculitis (LVV)
  - Takayasu arteritis (TAK)
  - Giant cell arteritis (GCA)
- Medium vessel vasculitis (MVV)
  - Polyarteritis nodosa (PAN)
  - Kawasaki disease (KD)
- Small vessel vasculitis (SVV)
  - Antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV)
    - Microscopic polyangiitis (MPA)
    - Granulomatosis with polyangiitis (Wegener's) (GPA)
    - Eosinophilic granulomatosis with polyangiitis (Churg-Strauss) (EGPA)
  - Immune complex SVV
    - Anti–glomerular basement membrane (anti-GBM) disease
    - Cryoglobulinemic vasculitis (CV)
    - IgA vasculitis (Henoch-Schönlein) (IgAV)
    - Hypocomplementemic urticarial vasculitis (HUV) (anti-C1q vasculitis)
- Variable vessel vasculitis (VVV)
  - Behçet's disease (BD)
  - Cogan's syndrome (CS)
- Single-organ vasculitis (SOV)
  - Cutaneous leukocytoclastic angiitis
  - Cutaneous arteritis
  - Primary central nervous system vasculitis
  - Isolated aortitis
  - Others
- Vasculitis associated with systemic disease
  - Lupus vasculitis
  - Rheumatoid vasculitis
  - Sarcoid vasculitis
  - Others
- Vasculitis associated with probable etiology
  - Hepatitis C virus–associated cryoglobulinemic vasculitis
  - Hepatitis B virus–associated vasculitis
  - Syphilis-associated aortitis
  - Drug-associated immune complex vasculitis
  - Drug-associated ANCA-associated vasculitis
  - Cancer-associated vasculitis
  - Others

*Reproduced from Jennette *et al*. [1] with permission of *John Wiley and Sons* (licence number 3316011094076).

1985 when van der Woude *et al*. described a new type of autoantibodies, which were found to bind to cytoplasmic components of neutrophils and monocytes [11]. These autoantibodies, now known as ANCA, were found to be present in the circulation of patients with GPA and the titre of ANCA was found to be related to disease activity. In the following years, it was discovered that ANCA recognize two major antigens, i.e., myeloperoxidase (MPO) [12] and proteinase 3 (PR3) [13], both of which are enzymes present in the azurophilic granules of neutrophils and monocytes.

Since the discovery of ANCA, their involvement in the pathogenesis of the disease has been questioned [11]. Although it is still unknown whether ANCA play a role in disease onset, their role in the effector phase of the disease is widely acknowledged [2]. The process by which ANCA-mediated neutrophil activation leads to vascular injury can be summarized as follows (figure 1). Due to a pre-existing pro-inflammatory environment, adhesion molecules are up-regulated on vascular endothelial cells. The pro-inflammatory cytokines also pre-activate neutrophils, leading to translocation of PR3 and MPO to the cell surface, where they become accessible for circulating ANCA. Pre-activated neutrophils roll over the activated endothelial cells and, upon

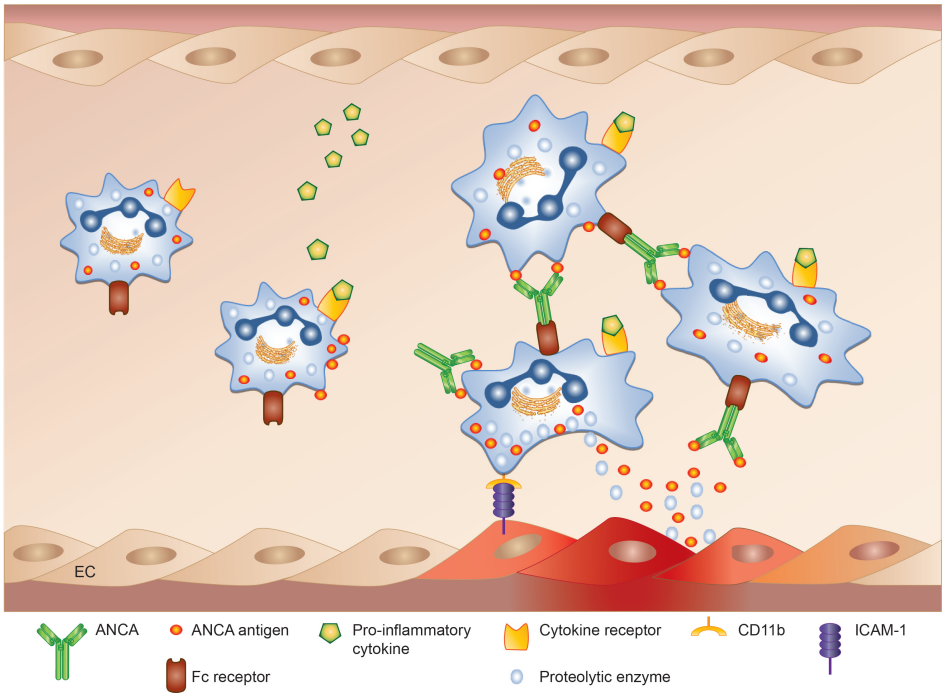


Figure 1. A schematic representation of ANCA-mediated vascular injury. Pro-inflammatory cytokines pre-activate neutrophils leading to ANCA antigen translocation to the cell surface. Neutrophils roll over pre-activated endothelium and in the presence of ANCA, become fully activated through Fc receptor interaction. Fully activated neutrophils produce reactive oxygen species and release proteolytic enzymes, which damage the endothelium.

ANCA binding, neutrophils become fully activated, resulting in firm adhesion [14], production of reactive oxygen species (ROS) and neutrophil degranulation [15, 16], which damages the endothelium and results in vasculitis.

AIM AND OUTLINE OF THE THESIS

Despite the fact that ANCA appear to play an important role in the effector phase of vasculitis, it is unclear how PR3- or MPO-specific autoreactive B cells are generated. However, T cells are thought to be significantly involved in this process because ANCA predominantly belong to the immunoglobulin (Ig) G1 and IgG4 subclasses [17]. Evidence also suggests that during active disease CD4+ effector memory T cells migrate to the site of the lesion [18], indicating their involvement in the inflammatory process. Furthermore, the use of B cell depletion therapy has advanced our understanding of the importance of B cells in the pathogenesis of AAV. It is now acknowledged that besides production of autoantibodies which trigger the inflammatory processes, B cells also contribute to the pathogenesis of AAV through autoantibody-independent mechanisms, although these are much less well understood [7].

The aim of this thesis was to study the mechanisms that contribute to the activation of B cells and the stimulation of autoantibody production in AAV. Furthermore, as B cells exert their effector functions also in an antibody-independent fashion, we investigated their regulatory function in patients with AAV and studied pathways which regulate cytokine production in primary human B cells.

In **chapter 2**, the literature on the pathophysiology of ANCA-associated vasculitides is reviewed with an emphasis on the role of adaptive immune mechanisms. In particular, the potential role of CD4+ T cells in the generation of PR3- and MPO-specific autoreactive B cells and the generation of high-affinity autoantibodies, as well as their possible involvement in vascular inflammation and granuloma formation is discussed.

Aberrations in T helper (Th) cell subsets, such as increased Th17 cells and dysfunctional regulatory T cells (T_{REGS}), are well-documented in AAV patients [19-23]. More recently, a Th cell subset which is competent of producing IL-21 has been described. This Th cell subset provides help to B cells during the germinal center reaction, aiding in the generation of high-affinity B cells and promoting antibody production [24]. Thus, in **chapter 3** we studied IL-21-producing CD4+ Th cells in patients with AAV and investigated the role of IL-21 in ANCA production *in vitro*. The involvement of IL-21 in ANCA production was studied in more depth in **chapter 4,** in which we investigated the factors that contribute to the production of ANCA *in vitro*. We tested the hypothesis that both endogenous and exogenous factors contribute to autoantibody production.

In contrast to the pathogenic effector functions of B cells, a subset of B cells has been proposed to exert immune-regulatory properties, primarily via the production of the anti-inflammatory cytokine IL-10 [25]. Therefore, in **chapter 5** we characterized the distribution of circulating B cell subsets, including 2 subsets, which have been proposed to have immune-regulatory properties. Furthermore, we studied the ability

of B cells to produce IL-10 and their capacity to suppress activation of other immune cells in healthy individuals and patients with AAV.

Emerging evidence suggests that B cell cytokine production is crucial in the regulation of immune-responses. Based on the cytokines they produce, B cells can possess a regulatory or pro-inflammatory function [26]. However, the mechanisms that determine skewing towards a particular phenotype are poorly characterized. Hence, in **chapter 6**, we further studied the regulation of pro- and anti-inflammatory cytokine production in primary human B cells. Because production of lineage-specific cytokines in CD4+ Th cells is epigenetically regulated [27, 28], we questioned whether cytokine expression in B cells is regulated in a similar manner. Specifically, we investigated if this process is regulated by histone/protein acetylases. For this reason, we tested the influence of the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) on B cell cytokine production. Finally, the work described in this thesis is summarized and discussed in **chapter 7**.

REFERENCES

1. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, Flores-Suarez LF, Gross WL, Guillevin L, Hagen EC, Hoffman GS, Jayne DR, Kallenberg CG, Lamprecht P, Langford CA, Luqmani RA, Mahr AD, Matteson EL, Merkel PA, Ozen S, Pusey CD, Rasmussen N, Rees AJ, Scott DG, Specks U, Stone JH, Takahashi K, Watts RA: **2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides.** *Arthritis Rheum* 2013, **65**(1):1-11.
2. Jennette JC, Falk RJ, Hu P, Xiao H: **Pathogenesis of antineutrophil cytoplasmic autoantibody-associated small-vessel vasculitis.** *Annu Rev Pathol* 2013, **8**:139-160.
3. Fauci AS, Haynes BF, Katz P, Wolff SM: **Wegener's granulomatosis: prospective clinical and therapeutic experience with 85 patients for 21 years.** *Ann Intern Med* 1983, **98**(1):76-85.
4. Flossmann O, Berden A, de Groot K, Hagen C, Harper L, Heijl C, Hoglund P, Jayne D, Luqmani R, Mahr A, Mukhtyar C, Pusey C, Rasmussen N, Stegeman C, Walsh M, Westman K, European Vasculitis Study Group: **Long-term patient survival in ANCA-associated vasculitis.** *Ann Rheum Dis* 2011, **70**(3):488-494.
5. Robson J, Doll H, Suppiah R, Flossmann O, Harper L, Hoglund P, Jayne D, Mahr A, Westman K, Luqmani R: **Damage in the anca-associated vasculitides: long-term data from the European Vasculitis Study group (EUVAS) therapeutic trials.** *Ann Rheum Dis* 2013, :.
6. Schonermarck U, Gross WL, de Groot K: **Treatment of ANCA-associated vasculitis.** *Nat Rev Nephrol* 2014, **10**(1):25-36.
7. Jones RB, Tervaert JW, Hauser T, Luqmani R, Morgan MD, Peh CA, Savage CO, Segelmark M, Tesar V, van Paassen P, Walsh D, Walsh M, Westman K, Jayne DR, European Vasculitis Study Group: **Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis.** *N Engl J Med* 2010, **363**(3):211-220.
8. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, Kallenberg CG, St Clair EW, Turkiewicz A, Tchao NK, Webber L, Ding L, Sejismundo LP, Mieras K, Weitzenkamp D, Ikle D, Seyfert-Margolis V, Mueller M, Brunetta P, Allen NB, Fervenza FC, Geetha D, Keogh KA, Kissin EY, Monach PA, Peikert T, Stegeman C, Ytterberg SR, Specks U, RAVE-ITN Research Group: **Rituximab versus cyclophosphamide for ANCA-associated vasculitis.** *N Engl J Med* 2010, **363**(3):221-232.
9. Cartin-Ceba R, Golbin JM, Keogh KA, Peikert T, Sanchez-Menendez M, Ytterberg SR, Fervenza FC, Specks U: **Rituximab for remission induction and maintenance in refractory granulomatosis with polyangiitis (Wegener's): ten-year experience at a single center.** *Arthritis Rheum* 2012, **64**(11):3770-3778.
10. Davies DJ, Moran JE, Niall JF, Ryan GB: **Segmental necrotising glomerulonephritis with antineutrophil antibody: possible arbovirus aetiology?** *Br Med J (Clin Res Ed)* 1982, **285**(6342):606.
11. van der Woude FJ, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, van der Giessen M, van der Hem GK, The TH: **Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis.** *Lancet* 1985, **1**(8426):425-429.
12. Falk RJ, Jennette JC: **Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis.** *N Engl J Med* 1988, **318**(25):1651-1657.
13. Niles JL, McCluskey RT, Ahmad MF, Arnaout MA: **Wegener's granulomatosis autoantigen is a novel neutrophil serine proteinase.** *Blood* 1989, **74**(6):1888-1893.
14. Radford DJ, Savage CO, Nash GB: **Treatment of rolling neutrophils with antineutrophil cytoplasmic antibodies causes conversion to firm integrin-mediated adhesion.** *Arthritis Rheum* 2000, **43**(6):1337-1345.
15. Falk RJ, Terrell RS, Charles LA, Jennette JC: **Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro.** *Proc Natl Acad Sci U S A* 1990, **87**(11):4115-4119.
16. Kettritz R, Jennette JC, Falk RJ: **Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils.** *J Am Soc Nephrol* 1997, **8**(3):386-394.

17. Brouwer E, Tervaert JW, Horst G, Huitema MG, van der Giessen M, Limburg PC, Kallenberg CG: **Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis and clinically related disorders.** *Clin Exp Immunol* 1991, **83**(3):379-386.
18. Abdulahad WH, Kallenberg CG, Limburg PC, Stegeman CA: **Urinary CD4+ effector memory T cells reflect renal disease activity in antineutrophil cytoplasmic antibody-associated vasculitis.** *Arthritis Rheum* 2009, **60**(9):2830-2838.
19. Abdulahad WH, Stegeman CA, Limburg PC, Kallenberg CG: **Skewed distribution of Th17 lymphocytes in patients with Wegener's granulomatosis in remission.** *Arthritis Rheum* 2008, **58**(7):2196-2205.
20. Nogueira E, Hamour S, Sawant D, Henderson S, Mansfield N, Chavele KM, Pusey CD, Salama AD: **Serum IL-17 and IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis.** *Nephrol Dial Transplant* 2010, **25**(7):2209-2217.
21. Wilde B, Thewissen M, Damoiseaux J, Hilhorst M, van Paassen P, Witzke O, Cohen Tervaert JW: **Th17 expansion in granulomatosis with polyangiitis (Wegener's): the role of disease activity, immune regulation and therapy.** *Arthritis Res Ther* 2012, **14**(5):R227.
22. Abdulahad WH, Stegeman CA, van der Geld YM, Doornbos-van der Meer B, Limburg PC, Kallenberg CG: **Functional defect of circulating regulatory CD4+ T cells in patients with Wegener's granulomatosis in remission.** *Arthritis Rheum* 2007, **56**(6):2080-2091.
23. Morgan MD, Day CJ, Piper KP, Khan N, Harper L, Moss PA, Savage CO: **Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells.** *Immunology* 2010, **130**(1):64-73.
24. Crotty S: **Follicular helper CD4 T cells (TFH).** *Annu Rev Immunol* 2011, **29**:621-663.
25. Mauri C: **Regulation of immunity and autoimmunity by B cells.** *Curr Opin Immunol* 2010, **22**(6):761-767.
26. Lund FE, Randall TD: **Effector and regulatory B cells: modulators of CD4(+) T cell immunity.** *Nat Rev Immunol* 2010, **10**(4):236-247.
27. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I: **Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells.** *Blood* 2008, **112**(6):2340-2352.
28. Wilson CB, Rowell E, Sekimata M: **Epigenetic control of T-helper-cell differentiation.** *Nat Rev Immunol* 2009, **9**(2):91-105.

CHAPTER 2

IMMUNE REGULATORY MECHANISMS IN ANCA-ASSOCIATED VASCULITIDES

Nikola Lepse, Wayel H. Abdulahad, Cees G.M. Kallenberg and Peter Heeringa

PUBLISHED
AUTOIMMUNITY REVIEWS 2011, 11(2):77-83

ABSTRACT

A group of primary vasculitides is associated with the presence of anti-neutrophil cytoplasmic autoantibodies (ANCA). In these diseases, the contribution of ANCA to disease pathogenesis has been studied extensively. Also, in patients with ANCA-associated vasculitides, the T lymphocyte compartment is dysregulated, as aberrant distribution and function of T cell subsets has been shown. In this review we will discuss the putative role of T lymphocytes in inflammation and granuloma formation, as well as the involvement of B cell compartments in the pathophysiology of ANCA-associated vasculitis.

INTRODUCTION

Vasculitides are systemic diseases characterized by inflammation of blood vessels. A specific subgroup of vasculitides is characterized by the presence of autoantibodies that are directed against lysosomal components of neutrophils and monocytes, and are therefore designated as anti-neutrophil cytoplasmic autoantibody (ANCA) associated vasculitides (AAV) [1]. The group of AAV comprises three disease entities; granulomatosis with polyangiitis (GPA, formerly known as Wegener's granulomatosis [2]), microscopic polyangiitis (MPA) and Churg-Strauss syndrome (CSS) [3]. A common pathological feature of AAV is necrotizing vasculitis of small vessels with a predilection for the kidneys and lungs. Additionally, GPA and CSS are also characterized by granulomatous inflammation affecting the respiratory tract, while this is not a common manifestation of MPA [1, 4]. Here we will briefly review the current knowledge on the pathophysiology of ANCA associated vasculitis with an emphasis on aberrations in the T and B lymphocyte compartments suggested to be involved in disease pathogenesis.

SPECIFICITY, PATHOGENICITY, AND ORIGIN OF ANCA

The antigenic specificity of ANCA is well characterized. Soon after the discovery of ANCA [5], myeloperoxidase (MPO) [6] and proteinase 3 (PR3) [7] were identified as the two major antigens [8]. PR3 and MPO are enzymes present in granules of neutrophils and monocytes. Normally PR3 and MPO are localized intracellularly, however, when neutrophils and monocytes are preactivated by proinflammatory cytokines (primed), these enzymes become expressed on the cell surface and are accessible for circulating ANCA [9]. According to the generally accepted mechanism of disease, primed neutrophils adhere to endothelial cells and become fully activated by ANCA, which leads to production of reactive oxygen species and release of granule proteolytic enzymes [10]. As neutrophilic infiltrates are commonly present in the early lesions of the affected blood vessels [11], the release of oxygen radicals and proteases from the infiltrated neutrophils further promotes vascular inflammation and injury [12].

In general, *in vitro* and *in vivo* studies have focused mainly on neutrophils as the major effector cells of AAV whereas the potential role of monocytes in AAV pathogenesis has received less attention. However, monocytes have been shown to be susceptible for ANCA mediated activation, resulting in elevated monocyte metabolic activity [13, 14]. Similar to neutrophils, ANCA-activated monocytes have been demonstrated to generate oxygen radicals and produce various cytokines that could contribute to the perpetuation of the inflammatory response [13]. Moreover, macrophages are an important cellular component of granulomatous inflammation in AAV suggesting involvement of monocytes in granuloma formation [15].

It has been questioned whether ANCA are pathogenic and direct evidence for a pathogenic role of ANCA in humans is not available. There is one intriguing case report of a neonate who developed pulmonary and renal disease after transplacental passage of MPO-ANCA suggesting development of AAV [16]. Additional indirect evidence indicating

a pathogenic role of ANCA comes from clinical trials that have demonstrated the efficacy of B cell depletion therapy [17, 18] and therapeutic plasma exchange [19]. In contrast, animal models (reviewed in [20]) have provided compelling evidence that MPO-ANCA can directly initiate vascular injury causing glomerulonephritis and pulmonary capillaritis [21]. Although to date no convincing animal model for PR3-AAV has been established, attempts are ongoing [22]. Importantly, no animal model for AAV is currently available that mimics the granulomatous inflammation which is a characteristic feature of GPA and CSS.

The aetiology of AAV remains unclear but clinical observations associate AAV with infections. In particular, a strong link has been established with *Staphylococcus aureus* (*S. aureus*) by demonstrating a substantially increased prevalence of chronic nasal carriage of *S. aureus* among AAV patients as compared to controls. Moreover, nasal carriage of *S. aureus* has been found to be an important risk factor for disease relapse [23] whereas treatment with co-trimoxazole significantly reduced disease relapse incidence [24]. Although these findings strongly support the contention that infections are involved in the aetiology of AAV, the underlying mechanisms are not fully understood.

Why tolerance to the ANCA antigens is broken in AAV is unknown but several potential mechanisms have been put forward. One of these mechanisms involves induction of autoimmunity through an immune response to a complementary peptide of the autoantigen that subsequently results in an immune response against the autoantigen via the idiotypic-anti-idiotypic pathway [25]. The nature and origin of the autoantigen complementary peptides are unknown but exogenous, infectious and endogenous sources have been proposed [25]. This theory of autoantigen complementarity has gained a lot of interest in the ANCA research community but is also controversial [26] because follow up studies from other laboratories could not completely confirm these observations [27].

More recently, it has been suggested that ANCA are part of the repertoire of natural autoantibodies (NAA) since MPO and PR3 specific NAA were found to be present in healthy individuals at low levels [28]. Further studies showed that NAA against MPO are of significantly lower titer and avidity for MPO compared to MPO-ANCA derived from AAV patients which could explain the non-pathogenic nature of NAA in healthy individuals. These interesting observations clearly require confirmation in larger cohorts. They do however suggest that dysregulation of the NAA-producing B cells may cause the generation of high affinity autoantibodies, resulting in autoimmune pathology although the underlying mechanisms are unknown [29].

T CELLS IN AAV

Although the pathogenic potential of ANCA, in particular MPO-ANCA, in causing acute vascular injury is well established, this by no means excludes an important role for T cell driven immune responses in the pathophysiology of AAV. A contribution of T cell mediated immune responses is highly likely because activated T cells can be readily detected in the inflammatory lesions observed in kidney, lung and nasal biopsies from

AAV patients and are a prominent component of the granuloma in GPA and CSS [30]. Moreover, several studies have reported that peripheral T cells are over-activated in AAV by demonstrating that various soluble T cell activation markers are elevated in plasma or serum of AAV patients [31, 32]. Also, ANCA antigen specific T cells have been detected in AAV [33, 34]. In AAV, T cell driven responses may be involved in disease pathogenesis at several levels including the induction, persistence and modulation of the autoreactive B cell response and regulation of the acute inflammatory response. Moreover, specific T cell subsets may also contribute to vascular inflammation directly [35]. In recent years, substantial progress has been made in the characterization of the T cell mediated responses in AAV as will be discussed below.

The involvement of CD4+ T effector memory cells

Studies in both mice and humans have demonstrated a crucial role for CD4+ T cells in the formation of granuloma. Mice deficient in CD4+ T cells have impaired ability to form granuloma upon infection with *Mycobacterium tuberculosis* [36], whereas patients with human immunodeficiency virus (HIV) associated tuberculosis exhibit less granuloma formation in lungs [37]. Our studies on the distribution of circulating CD4+ T-cell subpopulations in AAV-patients also demonstrate the involvement of CD4+ T-cells, particularly the effector memory T cells (CD4+ T_{EM}), in disease manifestation [38]. The effector memory T cell (T_{EM}) subset is defined by the lack of the lymph node homing receptor CCR7 and upregulation of chemokine receptors, such as CCR3 and CCR5 [39], that are necessary for migration to inflamed tissues. Therefore, these CD4+ T_{EM} cells fail to migrate to lymphoid organs, but can be directly recruited to sites of inflammation and may contribute to tissue injury and disease progression in AAV [40, 41].

Our studies demonstrated that the CD4+ effector memory T cell (T_{EM}) population is increased in peripheral blood of GPA patients in remission, but not in patients with active disease [38]. Further investigations showed that the decrease in circulating T_{EM} cells (compared to patients in remission) was in agreement with an increase in the number of CD4+ T_{EM} cells in urine sediments of patients with active renal disease [42]. This finding might reflect the role of CD4+ T_{EM} cells in renal injury during active disease. The fact that CD4+ T_{EM} cells migrate to the kidney during active disease, suggests that a specific stimulus which attracts these cells is being expressed by the target tissue. Indeed, Capraru and colleagues reported that CD4+ T_{EM} cells express the NKG2D receptor, whereas its ligand MICA was found to be expressed in granulomatous lesions of GPA patients [43]. MICA signaling via NKG2D receptor results in cytotoxic effects. NKG2D is usually expressed on NK cells, and T cells that express this receptor have a NK cell like phenotype. A recent study reported that CD4+NKG2D+ T cells isolated from GPA patients could induce endothelial damage *in vitro*. It was also shown that survival, expansion and cytotoxic properties of CD4+NKG2D+ T cells were dependant on interleukin 15 (IL-15) signaling [35], a cytokine mostly produced by macrophages. IL-15 levels were found to be elevated in GPA patients compared to healthy controls, suggesting that IL-15 may, via its effects on CD4+NKG2D+ T

cells, indirectly contribute to tissue injury in vasculitis. Furthermore regulatory T cells (T_{REG}) can inhibit T cell expansion [44] and control their transendothelial migration [44, 45]. As will be discussed later, evidence exists that the T_{REG} compartment in AAV is functionally defective. Thus, a lack of inhibitory mechanisms may promote T_{EM} cell migration to the inflamed sites as well.

The interplay between CD4+ T helper subsets and regulatory T cells

The major T helper (Th) effector cell types are distinguished according to surface markers expressed and the effector cytokines synthesized. To date these include Th1 (characterized by interferon-gamma (IFN-γ) production), Th2 (characterized by IL-4 production), Th17 (characterized by production of IL-17), regulatory T cells (distinguished by high expression of CD25 and FoxP3) and the most recently described T follicular helper (Tfh) cell lineage (known to produce IL-21) (reviewed in [46]). The involvement of different Th cell subsets in the pathogenesis of AAV has been suggested to depend on disease activity and whether the disease is localized, i.e. restricted to the respiratory tract, or generalized.

Characterization of T cells derived from the granulomatous lesions of AAV patients identified a predominant Th1 phenotype as these cells were found to produce IFN-γ, but not IL-4 [47]. In contrast, circulating IFN-γ levels did not significantly differ between AAV patients and healthy individuals suggesting that the skewing towards a Th1 response occurs locally [48]. Furthermore, elevated levels of sCD30 (a possible marker for Th2 responses) have been shown in plasma of AAV patients suggesting hyperactivation of the Th2 lineage, which has been associated with generalized disease [32].

Th17 cells are a recently defined Th cell subset characterized by secretion of IL-17A and other cytokines including IL-17F, IL-21 and IL-22. In recent years, Th17 cells have gained considerable interest as an important pathogenic effector subset contributing to inflammation and autoimmunity [49]. Our group has reported increased frequencies of Th17 cells in GPA patients in remission [50] and demonstrated that *in vitro* stimulation of peripheral blood mononuclear cells from these patients with PR3 led to preferential induction of IL-17 producing PR3 specific CD4+ cells. These observations were confirmed and extended in a study by Nogueira *et al*. demonstrating elevated IL-17 levels in serum from acute AAV patients [48]. Interestingly, this study also documented increased circulating levels of IL-23 in AAV patients compared to healthy individuals. IL-23 is a cytokine upstream from IL-17 that is crucial for Th17 lineage induction [48]. Also infectious agents can contribute to production of IL-23, as this cytokine is being produced by antigen presenting cells (APCs) when triggered by peptidoglycans and superantigens of *S. aureus* [51]. This suggests that the observed abnormalities in the CD4+ Th cell compartment in AAV patients may be partly attributed to deregulated expression of cytokines involved in Th lineage differentiation.

In the context of AAV, the reported effects of IL-17A on mobilization, recruitment and activation of neutrophils and stimulation of macrophages to produce IL-1β and tumor necrosis factor-alpha (TNF-α) are of particular interest [49, 52]. These pro-

inflammatory effects suggest that IL-17A may directly contribute to the acute vascular inflammatory response in ANCA associated vasculitis which is supported by the observation that IL-17A deficient mice are protected from disease development in a model of autoimmune anti-MPO mediated glomerulonephritis [53]. Interestingly, *in vitro* studies by Hoshino *et al.* [54] have demonstrated that mouse neutrophils in response to treatment with MPO-ANCA release IL-6, IL-17 and IL-23, providing an environment favouring Th17 differentiation. Collectively, these observations suggest an amplification loop in which ANCA mediated neutrophil activation contributes to the Th cell skewing towards a Th17 response whereas Th17 derived IL-17 promotes neutrophil recruitment and activation. However, whether such a mechanism is also operative in humans remains to be established. The association of PR3-ANCA positive GPA with increased nasal carriage of *S. aureus* seems to further strengthen the importance of Th17 responses in disease pathogenesis. Intranasal injection of peptidoglycans from *S. aureus* has been found to skew the immune response towards a Th17 phenotype in a IL-23 dependant manner [51] whereas *S. aureus* derived superantigens can induce expansion and IL-17 production in CD4+ T cells *in vitro* [50]. This suggests that *S. aureus* infection may drive the Th17 response in ANCA associated vasculitis. Finally, studies in systemic lupus erythematosus (SLE) have suggested that IL-17 may be involved in autoantibody production which adds to the supposed pathogenic effector properties of Th17 cells in AAV as well [55].

Another mechanism that might contribute to the skewing towards a Th17 response in AAV is the reported aberrant function of T_{REG} cells. Some reports suggest that under normal physiological conditions T_{REG} cells have a suppressive effect on Th17 cells [56], but this might not be the case in AAV due to a defective function of this subset. Initially, different research groups reported seemingly controversial results regarding the frequency of T_{REG} cells which were either found to be increased, decreased or unchanged in AAV patients compared to healthy controls [57-59]. These differences may be due, in part, to variations in methodology and gating strategies for $CD25^{high}$ T cells between these different studies. Importantly, despite these disagreements regarding the percentages of circulating T_{REG} cells, all studies reported an impaired functionality of the T_{REG} subset, demonstrating that T_{REGS} from AAV patients are not able to suppress proliferation of T cells. To date the underlying mechanisms for functional impairment of this subset remain unclear and need further investigation.

An important aspect of the T_{REG} subset is the plasticity of this population. Emerging evidence exists that T_{REG} cells are not terminally differentiated cells but, under specific circumstances, can convert into IL-17 producing cells [60, 61]. *In vitro* studies have shown that IL-1β mediates the skewing of human T_{REGS} towards IL-17 producing cells. In AAV patients, circulating levels of IL-1β are not different from those found in healthy controls [48] but *in situ* synthesis of IL-1β and TNF-α by infiltrated activated mononuclear cells in the kidneys of patients with ANCA-associated glomerulonephritis has been reported [62]. This is in accordance with the contention that conversion of T_{REG} towards IL-17-producing cells occurs locally at the site of inflammation.

Interestingly, a role for IL-17 in granuloma formation has also been suggested. In GPA, granuloma's have been shown to contain germinal center like structures [63] whereas the importance of Th17 cells and IL-17 in formation of autoreactive germinal centers *in vivo* has been demonstrated [64]. Therefore it is believed that IL-17 contributes to the formation of pseudo germinal centers in granulomatous tissue as well.

Another T helper cell subset proposed to be involved in the formation of germinal centers are Tfh cells. The main function of Tfh cells is to initiate differentiation of germinal center B cells towards memory and plasma cells, which are crucial processes for generation of high affinity antibodies. Tfh cell derived IL-21 promotes B cell proliferation, plasma cell formation and antibody production (reviewed in [65]). However, investigations on the involvement of Tfh cells in autoimmune pathology are still in its infancy. One study reported an association between high levels of Tfh cells in the circulation of SLE patients, autoantibody titer, and end-organ damage [66]. The authors suggested that increased frequencies of Tfh cells lead to formation of pathogenic germinal centers where high affinity autoantibody producing B cells arise. Interestingly, data from our laboratory indicate that percentages of Tfh cells are increased in patients with GPA as well [67]. As IL-21 is an important factor in regulation of antibody production and isotype switching [46], it is possible that Tfh cells also play a role in the development of pathogenic B cells but this certainly requires additional studies.

Taken together, imbalance in Th subsets may occur due to defective function of T_{REG} cells. Skewing towards Th17 and Tfh cells, therefore, may promote granuloma formation resulting in tissue injury and leading to vasculitis.

BEYOND AUTOANTIBODIES: THE EMERGING ROLE OF B CELLS IN AAV

In AAV, interest in B cell activation and regulation has increased considerably after the reports from clinical trials demonstrating the efficacy of the B cell depleting biological agent rituximab that targets CD20 [17, 18]. However, the exact role of B cells in AAV pathogenesis is not completely clear and data are limited. Obviously, in AAV, B cells are suggested to play a central role because these cells are the precursors for plasma cells that produce ANCA. However, B cells are also effective antigen presenting cells and can govern T cell responses by providing co-stimulatory signals and secretion of cytokines and growth factors. To date, these properties of B cells have received relatively little attention in AAV. In this context, an interesting case report has been described showing the efficacy of rituximab in a patient with ANCA-negative GPA [68]. In addition, analysis of the repopulating lymphocyte subsets after rituximab treatment in patients with SLE indicates that B cell depletion also markedly affects the T cell compartment (reviewed in [69]) including increased frequencies of T_{REG} cells. Thus, the role of B cells in vasculitis is most likely not limited to autoantibody production but remains to be fully characterized.

Phenotypic analysis of circulating B cells demonstrated that in patients with AAV and SLE a subset of B cells express elevated levels of CD19 [70] suggesting that

this might contribute to aberrant B cell receptor signaling possibly leading to B cell hyperactivation. Moreover, phenotypic analysis of the inflammatory infiltrate in renal biopsies of AAV patients has revealed significant B cell infiltration ranging from scattered B cells to organized B cell clusters [71]. Most of these intrarenal B cells were found to display a mature non-antibody producing phenotype with MHC class II expression suggesting a function of local antigen presenting cell.

Several groups have reported that the levels of B cell activating factor belonging to the TNF superfamily (BAFF) are elevated in AAV patients [32, 72]. BAFF is a known positive regulator of B cell survival, differentiation and proliferation. Animals overexpressing BAFF have uncontrolled B cell proliferation and develop autoimmune disease like manifestations [73]. In AAV controversial data exist regarding the correlation between circulating BAFF levels and ANCA titer. Nagai *et al*. have reported increased BAFF levels in active MPA patients which correlated strongly with MPO-ANCA titers [74]. In contrast, an inverse correlation between BAFF levels and ANCA titer has been demonstrated in GPA patients [75]. Interestingly, preliminary *in vitro* data by Holden *et al*. [76] indicate that neutrophil stimulation with ANCA leads to release of BAFF at sufficient levels to maintain B cell survival. This phenomenon might represent one of the mechanisms that lead to elevated BAFF levels in AAV patients. However, with respect to BAFF levels, our group reported no significant difference between ANCA-positive and ANCA-negative patients [32] and others even demonstrated higher levels in ANCA-negative patients [75]. This indicates that also ANCA-independent mechanisms are involved in BAFF overproduction. As the effect of BAFF on ANCA production is not known, further studies are needed to elucidate its exact role in AAV pathogenesis. This is particularly relevant in view of the possible use of BAFF inhibitors as future treatment.

Regulatory B cells (B_{REG} cells): modulators of CD4+ effector T cell responses

Already in the 1970s the first reports were published demonstrating that B cells can have immune suppressive properties [77]. More recent studies have provided additional evidence for the existence of such a regulatory subset of B cells in rodents [78, 79] and humans [80, 81]. These regulatory B cells (B_{REG}) are defined by a specific combination of phenotypic markers and cytokine secretion, and appear to be important in maintaining immunological tolerance through the synthesis of anti-inflammatory cytokines such as IL-10 and transforming growth factor-beta (TGFβ). In humans, the B_{REG} cell subset is characterized by the level of surface expression of CD24 and CD38 ($CD24^{high}CD38^{high}$) [80] whereas IL-10 production is considered to be the main mechanism mediating its suppressive function. It is not known how IL-10 production by B cells is regulated, as IL-10 production can be only attributed to approximately 5% of total B cells and no specific transcription factor for induction of IL-10 has been identified. The interaction of CD40 ligand (CD40L) and CD40 on B cells has been identified as one of the crucial pathways for IL-10 induction and indicates T cell involvement in the process [79]. In

addition, signaling via Toll-like receptors (TLRs) also has been reported to induce IL-10 production in B cells, which might seem controversial as signaling via TLRs is usually associated with proinflammatory responses. However, it has been suggested that the TLR mediated induction of regulatory B cells may be part of a normal response of the body to dampen inflammation and limit tissue damage [82].

It has been shown that B_{REG} cells are able to suppress IFN-γ production from CD4+ T cells in an IL-10 dependent manner [80, 83]. In addition, animal studies suggest that B_{REG} cells may have an important impact on the Th17 and T_{REG} cell compartments, as mice with a B cell lineage restricted IL-10 deficiency develop aggravated Ag-induced arthritis in conjunction with higher numbers of Th1/Th17 cells and a reduction in T_{REG} cells [84]. Interestingly, recent data indicate that T_{REG} cells lacking the IL-10 receptor do not suppress Th17 cells [85], indicating that IL-10 signaling in T_{REGS} is crucial for maintaining certain immune-suppressive functions of this subset. Theoretically, IL-10 production by B_{REG} cells could impact T_{REG} functionality which would add an additional level of regulation in the immune response. On the other hand, IL-10 producing B cells can suppress TNF-α production by activated monocytes [81]. This demonstrates an important mechanism by which B_{REG} cells are also capable of suppressing innate immune cell activation in humans which may be of relevance for many inflammatory diseases. Thus, B_{REG} cells serve as a potent negative regulator of autoimmunity and inflammation. B_{REG} cells in SLE patients are functionally impaired as they could not suppress IFN-γ production by CD4+ T cells [80]. However, the role and functionality of the B_{REG} subset in vasculitis is not yet established, but some indirect evidence exist for their possible role in AAV. In AAV patients higher levels of IL-10 in plasma have been detected compared to healthy controls [32] possibly as a means to suppress ongoing inflammation but whether this is derived from B_{REG} cells is unknown. Furthermore, lower levels of IL-10 at 3 months after diagnosis was associated with higher risk for disease relapse during the follow-up period [32]. Also a study by Hruskova *et al*. reported a relation between lower level of IL-10 and increased risk for disease relapse in AAV [86]. Moreover, elevated Th17 response and impaired T_{REGS} function in AAV patients could also be linked to a defect in B_{REG} function. In this case, future studies are needed to understand the potential role of B_{REG} cells in AAV.

The emerging anti-inflammatory functions of B_{REG} cells as discussed above raise the question whether (continuous) B cell depletion in AAV patients may also have unintended adverse effects.

CONCLUDING REMARKS

Given the established pathogenic effects of ANCA, AAV has been classified as an autoantibody mediated disease. However, as highlighted in this review, substantial evidence now exists pointing to an important role for cell mediated adaptive immune responses in disease pathogenesis as well (summarized in figure 1). In particular, dysregulation of T helper cell subsets has been demonstrated including skewing

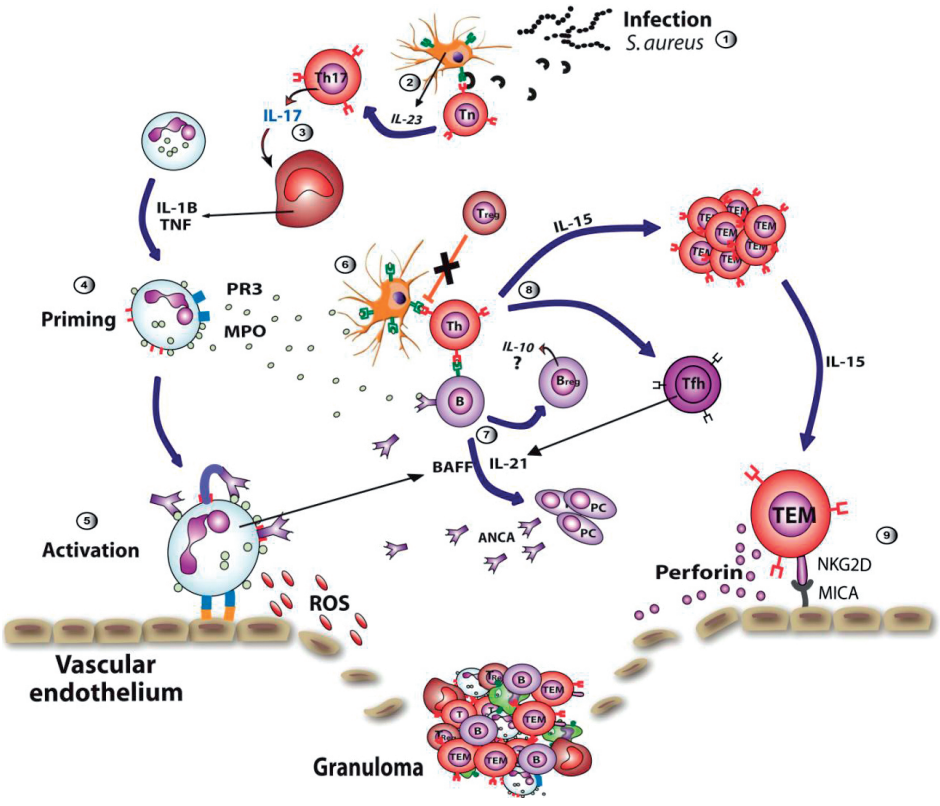


Figure 1. Overview of the innate and adaptive immune system components putatively involved in the development of ANCA-associated vasculitis. (1) Peptidoglycan and superantigens from *S. aureus* activate antigen presenting cells (APCs) to (2) produce IL-23, a cytokine necessary to skew and maintain a Th17 cell phenotype. (3) IL-17, released from activated Th17 cells, promotes the release of proinflammatory cytokines TNF-α and IL-1β from macrophages. (4) These proinflammatory cytokines induces release and translocation of the autoantigens (PR3 and MPO) on the neutrophil cell surface, on one hand, and promote the upregulation of adhesion molecules on both vascular endothelial cells and primed neutrophils, on the other hand. (5) Primed neutrophils are being recruited to the site of inflammaition and adhere to the vascular endothelium. Next, adherent neutrophils expressing PR3 or MPO on their surface become fully activated by ANCA leading to local production of reactive oxygen species and release of proteolytic enzymes that damage the endothelium. (6) Released PR3 or MPO from activated neutrophils, can be internalized and presented by APC to Th cells. Because of the impaired function of T_{REGS} (possible defect in B_{REGS}) in AAV, the autoreactive response can proceed, in which B-cells can differentiate into ANCA-producing plasma cells, whereas Th cells can skew towards Th17 and Tfh cells and expand into T_{EM} cells. (7) ANCA can also induce the release of BAFF from neutrophils. BAFF promotes the survival of autoreactive B cells, and together with IL-21 (produced by Tfh cells) synergize in stimulating plasma cell differentiation. (8) IL-15 which is overexpressed in AAV patients can promote the expansion of T_{EM} cells bearing the cytotoxic CD4+NKG2D+ phenotype. (9) During active disease, a ligand for NKG2D (that is MICA) becomes upregulated on endothelial cells. T_{EM} cells are attracted to the inflammatory areas and interact with MICA expressed by endothelial cells. This in turn enhances their cytotoxic function leading to endothelial cell killing in a perforin and granzyme dependent way and contributing to granuloma formation.

towards a Th17 response, a defective function of T_{REGS} cells and a possible role for Tfh cells. The underlying mechanisms responsible for inducing these aberrations within the T cell compartment and the interplay between them, are however, not completely clear.

The efficacy of B cell depletion therapy in AAV strongly indicates the involvement of B cells in pathological processes. In this context, it is also tempting to speculate that the reported effectiveness of rituximab in AAV is not only a direct consequence of B cell depletion, but is partly due to an indirect effect on the T cell effector arm of the (auto) immune response. We propose that a detailed phenotypic and functional analysis of T and B cell subsets in combination with autoantibody titers and disease activity in AAV patients after rituximab therapy will be instrumental in shedding light on these issues.

TAKE HOME MESSAGE

- Both the innate- and the adaptive- arms of the immune system are involved in the pathogenesis of AAV.
- In AAV, expansion of CD4+ T_{EM} cells and imbalance in Th cell subsets as well as impaired T_{REGS} function have been demonstrated. However, the cause and effect relationship (if any) between these observations is unclear.
- B_{REG} cells can play a critical role in maintaining immune homeostasis and preventing autoimmune inflammatory responses. Future studies on the involvement of B_{REG} cells in AAV are needed.
- The characterization of the lymphocyte population after rituximab treatment can be used to gain insights in whether the role of B cells in AAV autoimmune pathology is mainly limited to the humoral immune response or also affects cellular immunity.

REFERENCES

1. Jennette JC, Falk RJ: **Small-vessel vasculitis.** *N Engl J Med* 1997, **337**(21):1512-1523.
2. Falk RJ, Gross WL, Guillevin L, Hoffman GS, Jayne DR, Jennette JC, Kallenberg CG, Luqmani R, Mahr AD, Matteson EL, Merkel PA, Specks U, Watts RA, American College of Rheumatology, American Society of Nephrology, European League Against Rheumatism: **Granulomatosis with polyangiitis (Wegener's): an alternative name for Wegener's granulomatosis.** *Arthritis Rheum* 2011, **63**(4):863-864.
3. Jennette JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, Hagen EC, Hoffman GS, Hunder GG, Kallenberg CG: **Nomenclature of systemic vasculitides. Proposal of an international consensus conference.** *Arthritis Rheum* 1994, **37**(2):187-192.
4. Kallenberg CG: **Pathogenesis of ANCA-associated vasculitides.** *Ann Rheum Dis* 2011, **70 Suppl 1**:i59-63.
5. van der Woude FJ, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, van der Giessen M, van der Hem GK, The TH: **Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis.** *Lancet* 1985, **1**(8426):425-429.
6. Falk RJ, Jennette JC: **Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis.** *N Engl J Med* 1988, **318**(25):1651-1657.
7. Niles JL, McCluskey RT, Ahmad MF, Arnaout MA: **Wegener's granulomatosis autoantigen is a novel neutrophil serine proteinase.** *Blood* 1989, **74**(6):1888-1893.
8. Lochman I, Kral V, Lochmanova A, Lupac J, Cebecauer L: **ANCA in the diagnosis of neutrophil-mediated inflammation.** *Autoimmun Rev* 2011, **10**(6):295-298.
9. Harper L, Cockwell P, Adu D, Savage CO: **Neutrophil priming and apoptosis in anti-neutrophil cytoplasmic autoantibody-associated vasculitis.** *Kidney Int* 2001, **59**(5):1729-1738.
10. Kallenberg CG, Heeringa P, Stegeman CA: **Mechanisms of Disease: pathogenesis and treatment of ANCA-associated vasculitides.** *Nat Clin Pract Rheumatol* 2006, **2**(12):661-670.
11. Brouwer E, Huitema MG, Mulder AH, Heeringa P, van Goor H, Tervaert JW, Weening JJ, Kallenberg CG: **Neutrophil activation in vitro and in vivo in Wegener's granulomatosis.** *Kidney Int* 1994, **45**(4):1120-1131.
12. Hu N, Westra J, Kallenberg CG: **Dysregulated neutrophil--endothelial interaction in antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides: implications for pathogenesis and disease intervention.** *Autoimmun Rev* 2011, **10**(9):536-543.
13. Muller Kobold AC, Kallenberg CG, Tervaert JW: **Monocyte activation in patients with Wegener's granulomatosis.** *Ann Rheum Dis* 1999, **58**(4):237-245.
14. Wikman A, Fagergren A, Forslid J, Jacobson SH, Johansson SG, Lundahl J: **Antineutrophil cytoplasmic antibodies induce decreased CD62L expression and enhanced metabolic activity in monocytes.** *Scand J Immunol* 2003, **57**(2):179-184.
15. Mackiewicz Z, Rimkevicius A, Petersen J, Andersen CB, Dudek E, Vytrasova M, Konttinen YT: **Macrophages overloaded with tissue debris in Wegener's granulomatosis.** *Ann Rheum Dis* 2005, **64**(8):1229-1232.
16. Bansal PJ, Tobin MC: **Neonatal microscopic polyangiitis secondary to transfer of maternal myeloperoxidase-antineutrophil cytoplasmic antibody resulting in neonatal pulmonary hemorrhage and renal involvement.** *Ann Allergy Asthma Immunol* 2004, **93**(4):398-401.
17. Jones RB, Tervaert JW, Hauser T, Luqmani R, Morgan MD, Peh CA, Savage CO, Segelmark M, Tesar V, van Paassen P, Walsh D, Walsh M, Westman K, Jayne DR, European Vasculitis Study Group: **Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis.** *N Engl J Med* 2010, **363**(3):211-220.
18. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, Kallenberg CG, St Clair EW, Turkiewicz A, Tchao NK, Webber L, Ding L, Sejismundo LP, Mieras K, Weitzenkamp D, Ikle D, Seyfert-Margolis V, Mueller M, Brunetta P, Allen NB, Fervenza FC, Geetha D, Keogh KA, Kissin EY, Monach PA, Peikert T, Stegeman C, Ytterberg SR, Specks U, RAVE-ITN Research Group: **Rituximab versus cyclophosphamide for ANCA-associated vasculitis.** *N Engl J Med* 2010, **363**(3):221-232.

19. Pons-Estel GJ, Salerni GE, Serrano RM, Gomez-Puerta JA, Plasin MA, Aldasoro E, Lozano M, Cid J, Cervera R, Espinosa G: **Therapeutic plasma exchange for the management of refractory systemic autoimmune diseases: Report of 31 cases and review of the literature.** *Autoimmun Rev* 2011, :.
20. Heeringa P, Little MA: **In vivo approaches to investigate ANCA-associated vasculitis: lessons and limitations.** *Arthritis Res Ther* 2011, **13**(1):204.
21. Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, Maeda N, Falk RJ, Jennette JC: **Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice.** *J Clin Invest* 2002, **110**(7):955-963.
22. Little MA, Al-Ani B, Ren S, Al-Nuaimi H, Alpers CE, Savage C, Duffield JS: **Human anti-PR3 ANCA recapitulate systemic vasculitis in mice with a humanized immune system.** *Clin Exp Immunol* 2011, **164**(Suppl. 1):124.
23. Stegeman CA, Tervaert JW, Sluiter WJ, Manson WL, de Jong PE, Kallenberg CG: **Association of chronic nasal carriage of Staphylococcus aureus and higher relapse rates in Wegener granulomatosis.** *Ann Intern Med* 1994, **120**(1):12-17.
24. Stegeman CA, Tervaert JW, de Jong PE, Kallenberg CG: **Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. Dutch Co-Trimoxazole Wegener Study Group.** *N Engl J Med* 1996, **335**(1):16-20.
25. Pendergraft WF,3rd, Preston GA, Shah RR, Tropsha A, Carter CW,Jr, Jennette JC, Falk RJ: **Autoimmunity is triggered by cPR-3(105-201), a protein complementary to human autoantigen proteinase-3.** *Nat Med* 2004, **10**(1):72-79.
26. Preston G, Falk R: **Autoimmunity: Does autoantigen complementarity underlie PR3-ANCA AAV?** *Nat Rev Rheumatol* 2011, :.
27. Tadema H, Kallenberg CG, Stegeman CA, Heeringa P: **Reactivity against complementary proteinase-3 is not increased in patients with PR3-ANCA-associated vasculitis.** *PLoS One* 2011, **6**(3):e17972.
28. Cui Z, Zhao MH, Segelmark M, Hellmark T: **Natural autoantibodies to myeloperoxidase, proteinase 3, and the glomerular basement membrane are present in normal individuals.** *Kidney Int* 2010, **78**(6):590-597.
29. Xu PC, Cui Z, Chen M, Hellmark T, Zhao MH: **Comparison of characteristics of natural autoantibodies against myeloperoxidase and anti-myeloperoxidase autoantibodies from patients with microscopic polyangiitis.** *Rheumatology (Oxford)* 2011, **50**(7):1236-1243.
30. Wilde B, Thewissen M, Damoiseaux J, van Paassen P, Witzke O, Tervaert JW: **T cells in ANCA-associated vasculitis: what can we learn from lesional versus circulating T cells?** *Arthritis Res Ther* 2010, **12**(1):204.
31. D'Cruz D, Direskeneli H, Khamashta M, Hughes GR: **Lymphocyte activation markers and von Willebrand factor antigen in Wegener's granulomatosis: potential markers for disease activity.** *J Rheumatol* 1999, **26**(1):103-109.
32. Sanders JS, Huitma MG, Kallenberg CG, Stegeman CA: **Plasma levels of soluble interleukin 2 receptor, soluble CD30, interleukin 10 and B cell activator of the tumour necrosis factor family during follow-up in vasculitis associated with proteinase 3-antineutrophil cytoplasmic antibodies: associations with disease activity and relapse.** *Ann Rheum Dis* 2006, **65**(11):1484-1489.
33. Brouwer E, Stegeman CA, Huitema MG, Limburg PC, Kallenberg CG: **T cell reactivity to proteinase 3 and myeloperoxidase in patients with Wegener's granulomatosis (WG).** *Clin Exp Immunol* 1994, **98**(3):448-453.
34. Seta N, Kobayashi S, Hashimoto H, Kuwana M: **Characterization of autoreactive T-cell clones to myeloperoxidase in patients with microscopic polyangiitis and healthy individuals.** *Clin Exp Rheumatol* 2009, **27**(5):826-829.
35. de Menthon M, Lambert M, Guiard E, Tognarelli S, Bienvenu B, Karras A, Guillevin L, Caillat-Zucman S: **Excessive interleukin-15 transpresentation endows NKG2D+CD4+ T cells with innate-like capacity to lyse vascular endothelium in granulomatosis with polyangiitis (Wegener's).** *Arthritis Rheum* 2011, **63**(7):2116-2126.
36. Caruso AM, Serbina N, Klein E, Triebold K, Bloom BR, Flynn JL: **Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis.** *J Immunol* 1999, **162**(9):5407-5416.

37. Di Perri G, Cazzadori A, Vento S, Bonora S, Malena M, Bontempini L, Lanzafame M, Allegranzi B, Concia E: **Comparative histopathological study of pulmonary tuberculosis in human immunodeficiency virus-infected and non-infected patients.** *Tuber Lung Dis* 1996, **77**(3):244-249.
38. Abdulahad WH, van der Geld YM, Stegeman CA, Kallenberg CG: **Persistent expansion of CD4+ effector memory T cells in Wegener's granulomatosis.** *Kidney Int* 2006, **70**(5):938-947.
39. Lamprecht P, Erdmann A, Mueller A, Csernok E, Reinhold-Keller E, Holl-Ulrich K, Feller AC, Bruehl H, Gross WL: **Heterogeneity of CD4 and CD8+ memory T cells in localized and generalized Wegener's granulomatosis.** *Arthritis Res Ther* 2003, **5**(1):R25-31.
40. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A: **Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.** *Nature* 1999, **401**(6754):708-712.
41. Pepper M, Jenkins MK: **Origins of CD4(+) effector and central memory T cells.** *Nat Immunol* 2011, **12**(6):467-471.
42. Abdulahad WH, Kallenberg CG, Limburg PC, Stegeman CA: **Urinary CD4+ effector memory T cells reflect renal disease activity in antineutrophil cytoplasmic antibody-associated vasculitis.** *Arthritis Rheum* 2009, **60**(9):2830-2838.
43. Capraru D, Muller A, Csernok E, Gross WL, Holl-Ulrich K, Northfield J, Klenerman P, Herlyn K, Holle J, Gottschlich S, Voswinkel J, Spies T, Fagin U, Jabs WJ, Lamprecht P: **Expansion of circulating NKG2D+ effector memory T-cells and expression of NKG2D-ligand MIC in granulomaous lesions in Wegener's granulomatosis.** *Clin Immunol* 2008, **127**(2):144-150.
44. Tanriver Y, Martin-Fontecha A, Ratnasothy K, Lombardi G, Lechler R: **Superantigen-activated regulatory T cells inhibit the migration of innate immune cells and the differentiation of naive T cells.** *J Immunol* 2009, **183**(5):2946-2956.
45. Enarsson K, Lundin BS, Johnsson E, Brezicka T, Quiding-Jarbrink M: **CD4+ CD25high regulatory T cells reduce T cell transendothelial migration in cancer patients.** *Eur J Immunol* 2007, **37**(1):282-291.
46. Fazilleau N, Mark L, McHeyzer-Williams LJ, McHeyzer-Williams MG: **Follicular helper T cells: lineage and location.** *Immunity* 2009, **30**(3):324-335.
47. Csernok E, Trabandt A, Muller A, Wang GC, Moosig F, Paulsen J, Schnabel A, Gross WL: **Cytokine profiles in Wegener's granulomatosis: predominance of type 1 (Th1) in the granulomatous inflammation.** *Arthritis Rheum* 1999, **42**(4):742-750.
48. Nogueira E, Hamour S, Sawant D, Henderson S, Mansfield N, Chavele KM, Pusey CD, Salama AD: **Serum IL-17 and IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis.** *Nephrol Dial Transplant* 2010, **25**(7):2209-2217.
49. Ooi JD, Kitching AR, Holdsworth SR: **Review: T helper 17 cells: their role in glomerulonephritis.** *Nephrology (Carlton)* 2010, **15**(5):513-521.
50. Abdulahad WH, Stegeman CA, Limburg PC, Kallenberg CG: **Skewed distribution of Th17 lymphocytes in patients with Wegener's granulomatosis in remission.** *Arthritis Rheum* 2008, **58**(7):2196-2205.
51. Ivanov S, Bozinovski S, Bossios A, Valadi H, Vlahos R, Malmhall C, Sjostrand M, Kolls JK, Anderson GP, Linden A: **Functional relevance of the IL-23-IL-17 axis in lungs in vivo.** *Am J Respir Cell Mol Biol* 2007, **36**(4):442-451.
52. Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, Mineau F, Pelletier JP: **IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages.** *J Immunol* 1998, **160**(7):3513-3521.
53. Gan PY, Steinmetz OM, Tan DS, O'Sullivan KM, Ooi JD, Iwakura Y, Kitching AR, Holdsworth SR: **Th17 cells promote autoimmune anti-myeloperoxidase glomerulonephritis.** *J Am Soc Nephrol* 2010, **21**(6):925-931.
54. Hoshino A, Nagao T, Nagi-Miura N, Ohno N, Yasuhara M, Yamamoto K, Nakayama T, Suzuki K: **MPO-ANCA induces IL-17 production by activated neutrophils in vitro via classical complement pathway-dependent manner.** *J Autoimmun* 2008, **31**(1):79-89.
55. Crispin JC, Tsokos GC: **Interleukin-17-producing T cells in lupus.** *Curr Opin Rheumatol* 2010, **22**(5):499-503.

56. Fletcher JM, Lonergan R, Costelloe L, Kinsella K, Moran B, O'Farrelly C, Tubridy N, Mills KH: **CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis.** *J Immunol* 2009, **183**(11):7602-7610.

57. Abdulahad WH, Stegeman CA, van der Geld YM, Doornbos-van der Meer B, Limburg PC, Kallenberg CG: **Functional defect of circulating regulatory CD4+ T cells in patients with Wegener's granulomatosis in remission.** *Arthritis Rheum* 2007, **56**(6):2080-2091.

58. Morgan MD, Day CJ, Piper KP, Khan N, Harper L, Moss PA, Savage CO: **Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells.** *Immunology* 2010, **130**(1):64-73.

59. Rimbert M, Hamidou M, Braudeau C, Puechal X, Teixeira L, Caillon H, Neel A, Audrain M, Guillevin L, Josien R: **Decreased numbers of blood dendritic cells and defective function of regulatory T cells in antineutrophil cytoplasmic antibody-associated vasculitis.** *PLoS One* 2011, **6**(4):e18734.

60. Afzali B, Mitchell P, Lechler RI, John S, Lombardi G: **Translational mini-review series on Th17 cells: induction of interleukin-17 production by regulatory T cells.** *Clin Exp Immunol* 2010, **159**(2):120-130.

61. Abdulahad WH, Boots AM, Kallenberg CG: **FoxP3+ CD4+ T cells in systemic autoimmune diseases: the delicate balance between true regulatory T cells and effector Th-17 cells.** *Rheumatology (Oxford)* 2011, **50**(4):646-656.

62. Noronha IL, Kruger C, Andrassy K, Ritz E, Waldherr R: **In situ production of TNF-alpha, IL-1 beta and IL-2R in ANCA-positive glomerulonephritis.** *Kidney Int* 1993, **43**(3):682-692.

63. Kramer JA, Muller A, Herlyn K, Pitann S, Lamprecht P, Holl-Ulrich K, Feller AC, Gross WL, Gause A, Voswinkel J: **B lymphocyte differentiation in granulomatous tissues of the lung and the nasal mucosa in Wegener's granulomatosis: origin of anti-neutrophil cytoplasmic antibody formation?** *Z Rheumatol* 2007, **66**(5):421-429.

64. Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, Yi J, Guentert T, Tousson A, Stanus AL, Le TV, Lorenz RG, Xu H, Kolls JK, Carter RH, Chaplin DD, Williams RW, Mountz JD: **Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice.** *Nat Immunol* 2008, **9**(2):166-175.

65. Crotty S: **Follicular helper CD4 T cells (TFH).** *Annu Rev Immunol* 2011, **29**:621-663.

66. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, Manku H, Vyse TJ, Roncador G, Huttley GA, Goodnow CC, Vinuesa CG, Cook MC: **Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus.** *Arthritis Rheum* 2010, **62**(1):234-244.

67. Abdulahad WH, Lepse N, Tadema H, Huitema MG, Stegeman CA, Limburg PC, Heeringa P, Kallenberg CGM: **Increased frequency of circulating follicular helper T cells (T_{fh}) in patients with WG.** *Clin Exp Immunol* 2011, **164**(Suppl. 1):149.

68. Khan A, Lawson CA, Quinn MA, Isdale AH, Green MJ: **Successful Treatment of ANCA-Negative Wegener's Granulomatosis with Rituximab.** *Int J Rheumatol* 2010, **2010**:846063.

69. Lund FE, Randall TD: **Effector and regulatory B cells: modulators of CD4(+) T cell immunity.** *Nat Rev Immunol* 2010, **10**(4):236-247.

70. Culton DA, Nicholas MW, Bunch DO, Zhen QL, Kepler TB, Dooley MA, Mohan C, Nachman PH, Clarke SH: **Similar CD19 dysregulation in two autoantibody-associated autoimmune diseases suggests a shared mechanism of B-cell tolerance loss.** *J Clin Immunol* 2007, **27**(1):53-68.

71. Steinmetz OM, Velden J, Kneissler U, Marx M, Klein A, Helmchen U, Stahl RA, Panzer U: **Analysis and classification of B-cell infiltrates in lupus and ANCA-associated nephritis.** *Kidney Int* 2008, **74**(4):448-457.

72. Schneeweis C, Rafalowicz M, Feist E, Buttgereit F, Rudolph PE, Burmester GR, Egerer K: **Increased levels of BLyS and sVCAM-1 in anti-neutrophil cytoplasmatic antibody (ANCA)-associated vasculitides (AAV).** *Clin Exp Rheumatol* 2010, **28**(1 Suppl 57):62-66.

73. Mackay F, Schneider P: **Cracking the BAFF code.** *Nat Rev Immunol* 2009, **9**(1474-1741; 7):491-502.

74. Nagai M, Hirayama K, Ebihara I, Shimohata H, Kobayashi M, Koyama A: **Serum Levels of BAFF and APRIL in Myeloperoxidase Anti-Neutrophil Cytoplasmic Autoantibody-**

Associated Renal Vasculitis: Association with Disease Activity. *Nephron Clin Pract* 2011, **118**(4):c339-c345.

75. Bader L, Koldingsnes W, Nossent J: **B-lymphocyte activating factor levels are increased in patients with Wegener's granulomatosis and inversely correlated with ANCA titer.** *Clin Rheumatol* 2010, **29**(9):1031-1035.
76. Holden NJ, Williams JM, Harper L, Gordon J, Savage CO: **ANCA-stimulated neutrophils release BLyS and promote B cell survival *in vitro*.** *Clin Exp Immunol* 2011, **164**(Suppl. 1):137.
77. Katz SI, Parker D, Turk JL: **B-cell suppression of delayed hypersensitivity reactions.** *Nature* 1974, **251**(5475):550-551.
78. Wolf SD, Dittel BN, Hardardottir F, Janeway CA,Jr: **Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice.** *J Exp Med* 1996, **184**(6):2271-2278.
79. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM: **B cells regulate autoimmunity by provision of IL-10.** *Nat Immunol* 2002, **3**(10):944-950.
80. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, Mauri C: **CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients.** *Immunity* 2010, **32**(1):129-140.
81. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, Szabolcs PM, Bernstein SH, Magro CM, Williams AD, Hall RP, St Clair EW, Tedder TF: **Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells.** *Blood* 2011, **117**(2):530-541.
82. Mauri C: **Regulation of immunity and autoimmunity by B cells.** *Curr Opin Immunol* 2010, **22**(6):761-767.
83. Bouaziz JD, Calbo S, Maho-Vaillant M, Saussine A, Bagot M, Bensussan A, Musette P: **IL-10 produced by activated human B cells regulates CD4(+) T-cell activation in vitro.** *Eur J Immunol* 2010, **40**(10):2686-2691.
84. Carter NA, Vasconcellos R, Rosser EC, Tulone C, Munoz-Suano A, Kamanaka M, Ehrenstein MR, Flavell RA, Mauri C: **Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells.** *J Immunol* 2011, **186**(10):5569-5579.
85. Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, Jack RS, Wunderlich FT, Bruning JC, Muller W, Rudensky AY: **Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation.** *Immunity* 2011, **34**(4):566-578.
86. Hruskova Z, Rihova Z, Mareckova H, Jancova E, Rysava R, Zavada J, Merta M, Loster T, Tesar V: **Intracellular cytokine production in ANCA-associated vasculitis: low levels of interleukin-10 in remission are associated with a higher relapse rate in the long-term follow-up.** *Arch Med Res* 2009, **40**(4):276-284.

CHAPTER 3

INCREASED FREQUENCY OF CIRCULATING IL-21 PRODUCING TH-CELLS IN PATIENTS WITH GRANULOMATOSIS WITH POLYANGIITIS (GPA)

Wayel H. Abdulahad, Nikola Lepse, Coen A. Stegeman, Minke G. Huitema, Berber Doornbos-van der Meer, Henko Tadema, Abraham Rutgers, Pieter C. Limburg, Cees G.M. Kallenberg and Peter Heeringa

PUBLISHED
ARTHRITIS RESEARCH & THERAPY 2013, 15(3):R70

ABSTRACT

Introduction

The present study aimed to explore a possible role for IL-21 producing Th-cells in the immunopathogenesis of granulomatosis with polyangiitis (GPA).

Methods

Peripheral blood from 42 GPA patients in remission and 29 age-matched healthy controls (HCs) were stimulated *in vitro*, and the frequencies of IL-21 producing Th-cells were determined by flow cytometry. Since Th17 cells produce a low level of IL-21, IL-17 was also included in the analysis. Given that IL-21 is a hallmark cytokine for T follicular helper cells (T_{FH}), we next evaluated the expression of their key transcription factor BCL-6 by RT-PCR and flow cytometry. To investigate the effect of IL-21 on autoantibody production, PBMCs from GPA patients were stimulated *in vitro* with BAFF/IL-21 and total IgG and ANCA levels were measured in supernatants. In addition, the expression of IL-21-receptor on B cells was analyzed.

Results

Percentages of IL-21 producing Th-cells were significantly elevated in GPA patients compared to HCs, and were restricted to ANCA-positive patients. The expression of BCL-6 was significantly higher in ANCA-positive GPA patients, as compared with ANCA-negative patients and HCs. IL-21 enhanced the production of IgG and ANCA *in vitro* in stimulated PBMCs from GPA patients. No difference was found in the expression of the IL-21-receptor on B cells between ANCA-negative patients, ANCA-positive patients, and HCs.

Conclusion

The increased frequency of circulating IL-21 producing Th-cells in ANCA-positive GPA patients and the stimulating capacity of IL-21 on ANCA-production suggest a role for these cells in the immunopathogenesis of GPA. Blockade of IL-21 could constitute a new therapeutic strategy for GPA.

INTRODUCTION

Granulomatosis with polyangiitis (GPA) is an autoimmune vasculitis of small- to medium-sized blood vessels, associated with the presence of circulating anti-neutrophil cytoplasmic autoantibodies (ANCA) that are mainly directed against proteinase 3 [1-3]. Histopathologically, GPA is characterized by granulomatous inflammation and pauci-immune vasculitis, including necrotizing crescentic glomerulonephritis.

Although the production of ANCA is directly attributable to autoreactive B cells, there is extensive evidence that T cells play a critical role in GPA as well. The immunoglobulin (Ig) G subclass distribution of ANCA, with a preponderance of the IgG1 and IgG4 subclasses, suggests a T cell–dependent immune response [4]. Infiltrating T cells in granulomatous lesions and persistent T cell activation have been observed in GPA patients [5, 6]. In addition, an aberrant T cell phenotype and impaired regulatory T cell function are also reported in GPA patients in remission [7-9], suggesting that even during remission, the immune system is dysregulated. Moreover, T-helper (Th) cell polarization with an increase in Th17 cells has been demonstrated [10, 11]. Th17 cells and their cytokine IL-17 have been shown to play a critical role in many inflammatory diseases. In addition to IL-17, Th17 cells can produce IL-21, a cytokine that is largely responsible for B cell class switching and antibody production, and which induces differentiation of B cells towards plasma cells by synergizing with B cell activating factor (BAFF) [12, 13]. Therefore, it is conceivable that IL-21 may contribute to the production of pathogenic autoantibodies in GPA.

Multiple studies in animal models indicate a pivotal role of IL-21 in the pathogenesis of autoimmune diseases. Studies in arthritis models have shown that blockade of IL-21 activity reduces joint inflammation and destruction [14]. Subsequent investigations demonstrated that blocking of the IL-21 pathway reduces levels of anti-dsDNA autoantibodies and prevents renal disease in mouse models of systemic lupus erythematosus (SLE) [15]. In addition, mice deficient in IL-21-receptor expression were found to be protected to a large extent against the development of inflammatory bowel disease (IBD) and type-I diabetes [16, 17]. Interestingly, recent genome-wide association studies have provided convincing evidence that genetic variants in the region on chromosome 4q27 that harbor the IL-21 and IL-2 genes are associated with chronic inflammatory disorders, including SLE, IBD and psoriasis [18-20]. Thus, IL-21 seems to play an important role in autoimmune diseases in general and could constitute a novel target for therapy.

IL-21 is produced mainly by activated $CD4^+$ Th-cells. Recent studies have demonstrated that IL-21, besides its production by Th17 cells, is predominantly secreted by a distinct Th-cell lineage, termed follicular helper T cells (T_{FH}) that express the transcription factor BCL-6 and are considered to be specialized providers of B cell help [21]. Expansion of circulating T cells resembling T_{FH} cells has been reported in patients with SLE and in patients with rheumatoid arthritis [22-24]. To date, no study has investigated the role of IL-21-producing Th-cells in GPA. Therefore, this study aimed to assess the frequency of IL-21 producing Th-cells, and to evaluate whether T_{FH}

cells or Th17 cells are the major source of IL-21 in GPA patients. For this purpose, we examined the expression of both IL-21 and IL-17 in circulating $CD4^+$ T cells of patients with GPA. To improve our understanding of the role of IL-21-producing Th-cells in autoantibody production we assessed their frequencies in ANCA-positive and ANCA-negative patients, and studied effects of IL-21 on Ig and ANCA production *in vitro*.

METHODS

Study population

Forty-two patients with GPA and 29 age- and sex-matched healthy controls (HC) (18 male, 11 female, mean age 56 (SD ±13) years, range 26 to 72 years, *P*= 0.16) were included in this study. The diagnosis of GPA was established according to the definitions of the Chapel Hill Consensus Conference and patients fulfilled the classification criteria of the American College of Rheumatology (ACR) [25, 26]. Only patients without clinical signs and symptoms of active vasculitis and considered to be in complete remission, as indicated by a score of zero on the Birmingham Vasculitis Activity Score (BVAS), were included in the study [27]. Serostatus for ANCA was followed for several months in all patients, and patients with a stable serostatus for ANCA (positive or negative) for at least 3 months were included in this study. Based on these criteria, 23 patients were positive for PR3-ANCA, whereas 19 were ANCA-negative. There was history of generalized disease including renal involvement in 27 patients, and 15 patients had localized disease, which had been confined to the upper and/or lower respiratory tract. None of the patients and controls had infection at the time of sampling. Eleven GPA patients (eight ANCA-positive, and three ANCA-negative) were treated with maintenance immunosuppressive therapy at the time of blood sampling. Four of them were treated with only azathioprine (25 to100 mg/day), one patient with mycophenolate mofetil (1500 mg/day), and six patients received prednisolone (5 to 10 mg/day) in combination with azathioprine (125 mg/day). Participants in rituximab trials were excluded from the present study. The main clinical and laboratory data from the patients are summarized in table 1. All patients and healthy individuals provided informed consent and the study was approved by the local Medical Ethics Committee of the University Medical Centre Groningen, University of Groningen (NL).

Measurement of ANCA titres and specificity

ANCA titers were measured by indirect immunofluorescence (IIF) on ethanol-fixed human granulocytes according to standard procedures as previously described [28]. ANCA titers higher than 1:40 were considered positive. ANCA antigenic specificity was determined using an in-house capture ELISA as described before [29, 30]. Briefly, a 96-well plate was coated with goat-anti-mouse Ig for 48 hours. After washing, plates were incubated with mouse monoclonal antibody against human PR3 for 2 hours. After washing, the plate was incubated overnight at 4 °C with an extract of human azurophilic granules which were isolated from neutrophils of healthy donors. Further,

Table 1. Clinical and laboratory characteristics of patients with granulomatosis with polyangiitis (GPA) at the time of blood sampling

Characteristic	Value
Patients, number	42
Male/female, number	26/16
Age, mean ±SD (range, years	59 ±14 (28-81)
Localized/generalized GPA, number	15/27
Positive/negative for PR3-ANCA†, number	23/19
Receiving/not receiving treatment††, number	11/31
Number of relapses, median (range)	0 (0-5)
Disease duration, median (range), months	112 (20-334)

† Patients were considered to be ANCA-positive when ANCA-titres by IIF were greater than 1:40. †† Four patients were treated with only azathioprine, one with mycophenolate mofetil, and six patients received prednisolone in combination with azathioprine. GPA, granulomatosis with polyangiitis; PR3, proteinase 3; ANCA, anti-neutrophil cytoplasmic autoantibody.

serial dilutions of serum (with a starting dilution of 1:100) were incubated for 1 hour. The plate was washed, and the captured antibodies were detected with purified $F(ab)_2$ goat-anti-human IgG conjugated to alkaline phosphatase. P-nitrophenyl-phosphate disodium was used as a substrate, and the optical density was measured at 405 nm.

Antibodies used in flow cytometry

The following conjugated antibodies were used in flow cytometry: allophycocyanin (APC)-conjugated anti-CD3 (clone UCHT1), peridin chlorophyll protein (PerCP)-conjugated anti-CD8 (clone SK1), phycoerythrin (PE)-conjugated anti-IL-21-receptor (clone 17A12), and PerCP-conjugated anti-CD19 (clone 4G7), all purchased from Becton & Dickinson (Amsterdam, the Netherlands); PE-conjugated anti-IL-21 (clone eBio3A3-N2), Alexa Fluor® 488 (A488)-conjugated anti-IL-17 (clone eBio64DEC17), and A488-conjugated anti-FoxP3 (clone PCH101), all purchased from eBioscience (San Diego, CA, USA); PE-conjugated anti-BCL-6 (clone IC5046P) was obtained from R&D Systems (Minneapolis, MN, USA).

Sample preparation and *in vitro* stimulation

Lithium-heparinized venous blood was obtained from patients and healthy donors. Immediately after sampling, 400 µL blood was mixed with 400 µL RPMI 1640 (Cambrex Bio Science, Verviers, Belgium), supplemented with 50 µg/mL gentamycin (Gibco, Scotland, UK), and aliquoted in two 5 mL polypropylene tubes (BD Biosciences, Amsterdam, the Netherlands) (400 µL per tube). Diluted blood samples were stimulated for 4 hours with 50 ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich, Steinheim, Germany) and 2 nM calcium ionophore (Ca-Io; Sigma-Aldrich). As a negative control, one sample was kept in medium only without stimulation. For inhibiting cytokine release from the cells, 10 µg/mL of brefeldin A (BFA; Sigma-Aldrich) was added to each sample.

Intracellular staining for cytokines

After stimulation, cells were washed in wash buffer [PBS, 5% fetal bovine serum (FBS), 0.1% sodium azide (Merk, Germany)] and stained with PerCP-conjugated anti-CD8 and APC-conjugated anti-CD3 for 15 minutes at room temperature. Cells were fixed with 100 µL *Reagent A* (Caltag/Invitrogen, Breda, the Netherlands) for 10 minutes. After washing, the pellet was resuspended in 100 µL permeabilization *Reagent B* (Caltag/Invitrogen) and labelled with A488-conjugated anti-IL-17 and PE-conjugated anti-IL-21 for 20 minutes in the dark. After staining, the cells were washed and immediately analyzed on FACSCalibur flow cytometer (Becton & Dickinson). Data were collected for 2 x 10^5 cells, and plotted using the Win-List software package (Verity Software House Inc, ME, USA). Because stimulation reduces surface expression of CD4 on T cells, $CD4^+$T cells were identified indirectly by gating on CD3-positive and CD8-negative lymphocytes. Gated $CD4^+$ T cells were further displayed as a dot plot for evaluation of intracellular cytokine production. The unstimulated control sample was used as a guide for setting the linear gates to discriminate positive and negative populations.

Intracellular staining for transcription factors

Peripheral blood mononuclear cells (PBMCs) from GPA patients and HCs were prepared from heparinized venous blood by density-gradient centrifugation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Cells recovered from the gradient interface were washed twice in PBS and stained for BCL-6 and FoxP3 according to the manufacturer's instructions (eBioscience staining set for transcription factors). Briefly, PBMCs were adjusted to 1 x 10^6 cells in 100 µL and incubated with appropriate concentration of APC-conjugated anti-CD3 and PerCP-conjugated anti-CD8 for 30 minutes at 4 °C in the dark, followed by fixation and permeabilizaion in Fix/Perm buffer (eBioscience) for 45 minutes. Cells were then washed twice with 1X permeabilization buffer (eBioscience) and stained with PE-conjugated anti-BCL-6 and A488-conjugated anti-FoxP3. After incubation for 30 minutes in the dark, the cell suspension was washed and immediately analyzed on the FACSCalibur flow cytometer (Becton & Dickinson). Lymphocytes were gated by forward and side scatter patterns and plotted using the Win-List software package (Verity Software House Inc). Isotype matched control antibodies of irrelevant specificity were obtained from eBioscience and R&D Systems.

Immunofluorescent surface staining for IL-21R on B cells

Fresh blood samples from GPA patients and HCs were labeled with PE-conjugated anti-IL-21R, and PerCP-conjugated anti-CD19 for 15 minutes in the dark. Cells were successively treated with 2 mL diluted FACS lysing solution (BD, Amsterdam, the Netherlands) for 10 minutes and then washed twice in wash buffer and immediately analyzed by flow cytometry.

RNA isolation and real-time reverse transcription (RT)-PCR

Erythrocytes were lysed and leukocytes were fixed and washed twice in 1% BSA. RNA was isolated from total leukocytes with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. DNAse treatment (Ambion, Huntingdon, Cambridgeshire, UK) was performed and subsequently cDNA was synthesized using M-MLV reverse transcriptase and oligo (dT) 14 to 18 as primer. For measurement of mRNA for BCL-6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1 µl of cDNA in triplicate was used for amplification by the Taqman RT-PCR system (ABI Prism 7900HT Sequence Detection System, Applied Biosystems, Foster City, CA, USA) with specific Taqman primers/probes (BCL-6 (Hs 00153368_m1) and GAPDH (Hs 99999905_m1), Applied Biosystems). Amplification was performed using standard conditions and calculations of fold induction were performed. We normalized gene expression to GAPDH and expressed values relative to control using the CT method.

Cell stimulation and total IgG production

PBMCs recovered from the gradient interface were washed twice in PBS and adjusted to 10^6 cells/mL in RPMI 1640 (Lonza, Switzerland) supplemented with 10% FCS (Lonza, Basel, Switzerland) and 50 µg/mL gentamicin (GIBCO, Invitrogen). Cells were cultured in the presence of 100 ng/mL rhIL-21 (ImmunoTools GmbH, Friesoythe, Germany) and/or 100 ng/mL rhBAFF (PeproTech, USA) for 12 days at 37 °C with 5% CO_2. After 12 days, culture supernatants were collected and total IgG was measured using an in-house ELISA as described previously [31]. Briefly, Costar 96-well ELISA plates were coated with 2 mg/mL goat anti-human-Ig antibody (Southern Biotech, Birmingham, AL, USA) in carbonate buffer (0.01M, pH 9.6). Plates were washed with washing buffer (0.025 M Tris-HCl, 0.15 M NaCl, 0.05% Tween-20) and blocked for 1 h with blocking/incubation-buffer (0.05 M Tris-HCl, 0.3 M NaCl, 0.05% Tween-20, 1% BSA). Cell culture supernatants were diluted in incubation buffer. Purified human IgG with a known concentration was used as a standard sample. The bound IgG was detected with goat-anti-human-IgG antibody conjugated with alkaline phosphatase (Sigma, St Louis, MO, USA). P-nitrophenyl-phosphate disodium was used as substrate and optical density was read at 405 nm using an Emax microplate reader (Molecular Devices, Silicon Valley, CA, USA).

Measurement of *in vitro* production of PR3-ANCA

In vitro PR3-ANCA IgG production in PBMC culture supernatants was measured by Phadia ImmunoCAP® 250 analyzer (Thermo Fisher Scientific) using ELiA™ PR3, and the levels of PR3-ANCA IgG production were expressed in response units (RU).

Statistical analysis

Data are presented as median values, unless stated otherwise. The nonparametric Mann-Whitney U test was used to compare data from patients with those of healthy controls. The Wilcoxon matched pairs test was used for intra-individual comparison.

Correlations were assessed using Spearman's rank correlation coefficient. Two-tailed *P*-values lower than 0.05 were considered statistically significant.

RESULTS

Increased percentage of circulating IL-21^{+}IL-17^{-} cells in ANCA-positive GPA patients compared to ANCA-negative patients and healthy controls

We initially determined the frequency of IL-21 producing CD4 T cells in the peripheral blood of GPA patients (n= 42) and HCs (n= 29) after *in vitro* stimulation. The percentage of circulating IL-21^{+} Th-cells was significantly higher in GPA patients as compared with the control group (Figure 1B). Of note, Th17 cells may produce IL-21 in addition to their signature cytokine IL-17. Since Th17 cells are increased in GPA patients [10, 11], we next extended our analysis to investigate whether increased IL-21^{+} Th-cells in GPA patients resulted from an increase in Th17 cells. To this end, IL-17 staining was included in the analysis to determine what percentage of the total IL-21^{+} Th-cells are Th17 cells. Using this approach, we assessed the frequency of IL-21^{+}IL-17^{-}, IL-21^{+}IL-17^{+}, and IL-21^{-}IL-17^{+} cells within the CD4 T cells in GPA patients and HCs. As shown in figure 1 (C, D and E), GPA patients in remission had a significantly higher percentages of circulating IL-21^{+}IL-17^{-}, IL-21^{+}IL-17^{+}, and IL-21^{-}IL-17^{+} cells as compared with the control group. However, the majority of circulating CD4^{+} T cells that produced only IL-21 were distinct from Th17 cells, that is negative for IL-17. To assess the possible role of IL-21^{+}IL-17^{-} Th-cells in ANCA production, we compared their percentage between patients who were ANCA-positive (n= 23; IIF titer >1:40) or ANCA-negative (n = 19) at the time of inclusion. Significant increases in the frequencies of IL-21^{+}IL-17^{-} Th-cells were observed in ANCA-positive patients in comparison with HCs and ANCA-negative patients, whereas no significant difference was found between ANCA-negative patients and HCs (Figure 1F). In contrast, the percentages of IL-21^{+}IL-17^{+} and IL-21^{-}IL-17^{+} Th-cells in ANCA-positive GPA patients did not differ from those in ANCA-negative GPA patients (Figure 1G and H). These results suggest that persistence of IL-21^{+}IL-17^{-} Th-cells during remission plays a role in the ongoing humoral autoimmune response in ANCA-positive GPA patients.

To rule out the possibility that the increased proportion of IL-21^{+}IL-17^{-} Th-cells in GPA patients was the result of current treatment, the ANCA-positive patient group was divided into treated and untreated patients, and the percentages of IL-21^{+}IL-17^{-} Th-cells were compared. No significant differences were observed between treated and untreated patients (data not shown). We also compared the percentage of IL-21^{+}IL-17^{-} Th-cells between currently untreated ANCA-positive patients with a history of generalized disease and those with localized disease. No difference was found between these patient groups (data not shown).

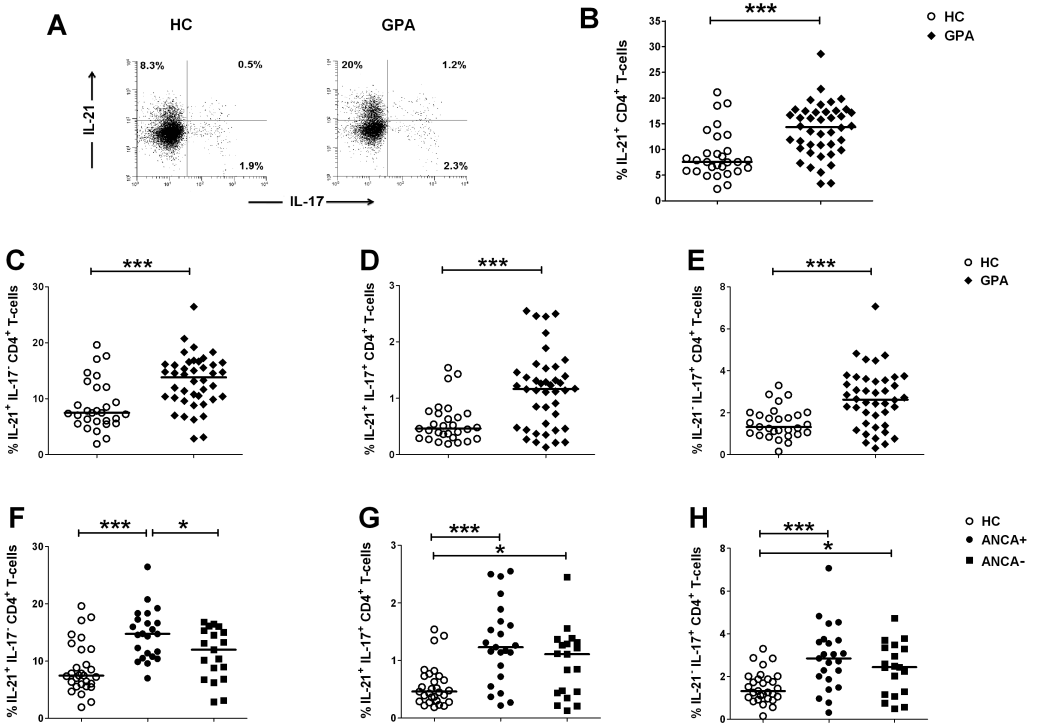


Figure 1. Multiparameter flow cytometric detection of IL-21 and IL-17 in circulating CD4⁺ T cells in patients with granulomatosis with polyangiitis (GPA) and healthy controls (HCs). Whole blood from GPA patients and HCs was stimulated with phorbol myristate acetate /Ca-ionophore and analyzed for intracellular IL-21 and IL-17 cytokine expression. Representative flow cytometry plots of IL-21 versus IL-17 expression in stimulated CD4⁺ T cells from a GPA patient (right plot) and an age- and sex-matched HC (left plot) (**A**). Value in each gate represents the percentages of cytokine producing cells. Percentages of total IL-21 producing Th-cells in peripheral blood of GPA patients (n= 42) and HCs (n= 29) (**B**). Percentages of circulating IL-21⁺IL-17⁻, IL-21⁺IL-17⁺ and IL-21⁻IL-17⁺ cells within the CD4⁺ T cells in all GPA patients and HCs (**C-E**), or in ANCA-positive (n= 23) and ANCA-negative (n= 19) GPA patients (**F-H**). Horizontal lines represent the median percentage. *P*-values were calculated using the nonparametric Mann-Whitney U-test. * $P < 0.05$; *** $P < 0.0005$.

Increased frequencies of IL-21^{+}IL-17^{-} Th-cells correlate positively with Th17 response

It has been reported that IL-21 is a key factor regulating the differentiation of naïve CD4^{+} T cells into Th17 cells [32, 33]. In order to analyze this relationship, we tested correlation between percentages of IL-21^{+}IL-17^{-} Th-cells and percentages of terminally differentiated Th17 cells (IL-21^{-}IL-17^{+}) in GPA patients (n= 42) and HCs (n= 29). Interestingly, a significant positive correlation was observed between IL-21^{+}IL-17^{-} Th-cells and IL-21^{-}IL-17^{+} Th-cells in both GPA patients and HCs (r= 0.58, *P*< 0.0001 and r= 0.37, *P*= 0.04, respectively) (Figure 2A and B).

Increased frequencies of BCL-6^{+} CD4^{+} T cells in peripheral blood of ANCA-positive GPA patients

Since IL-21 is not the only marker for T_{FH} cells, we further characterized the identity of circulating IL-21 producing cells by analyzing BCL-6 expression, which is considered a master regulator and specific transcription factor for T_{FH} cells [34, 35]. To this end, the expression of BCL-6 mRNA was assessed in circulating leukocytes from GPA patients and HCs by real-time RT-PCR. Restricted numbers of patients and controls were included in this analysis due to insufficient cell numbers. Patients with ANCA-positive GPA (n= 10) had a significantly higher expression of mRNA BCL-6 than ANCA-negative patients (n= 6) and HCs (n= 11) (Figure 3B). In addition, intracellular FACS-staining for BCL-6 within circulating CD4+ T cells confirmed the increased BCL-6 expression in ANCA-positive GPA patients (Figure 3A and C). We have also analyzed the MFI (mean fluorescence intensity) of BCL-6 expression in CD4^{+} T cells from patients and HCs and found that the expression level of BCL-6 per Th-cell in GPA patients was similar to that in HCs (data not shown). Thus, BCL-6 expression is increased in GPA patients due to increased frequencies of circulating BCL-6^{+} CD4^{+} T cells.

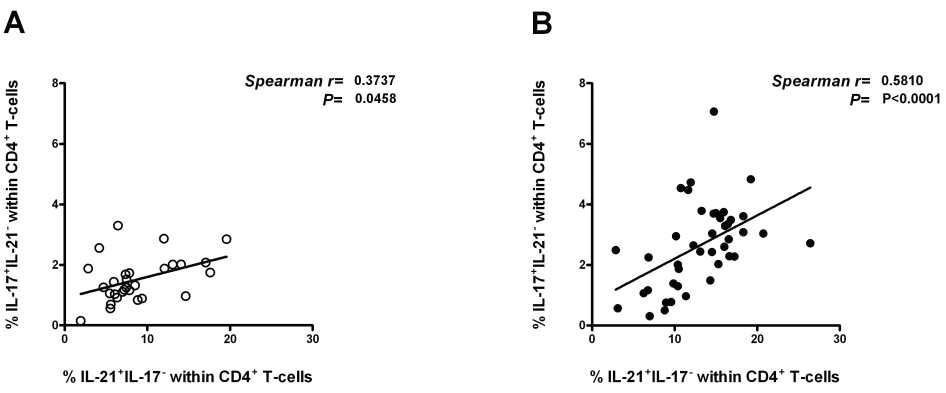


Figure 2. Correlation between the percentages of IL-21^{+}IL-17^{-} cells and IL-21^{-}IL-17^{+} cells within the CD4^{+} T cells in peripheral blood of healthy controls (HCs) (A), and patients with granulomatosis with polyangiitis (GPA) (B). Spearman rank correlation coefficients (r) and *P* values are given.

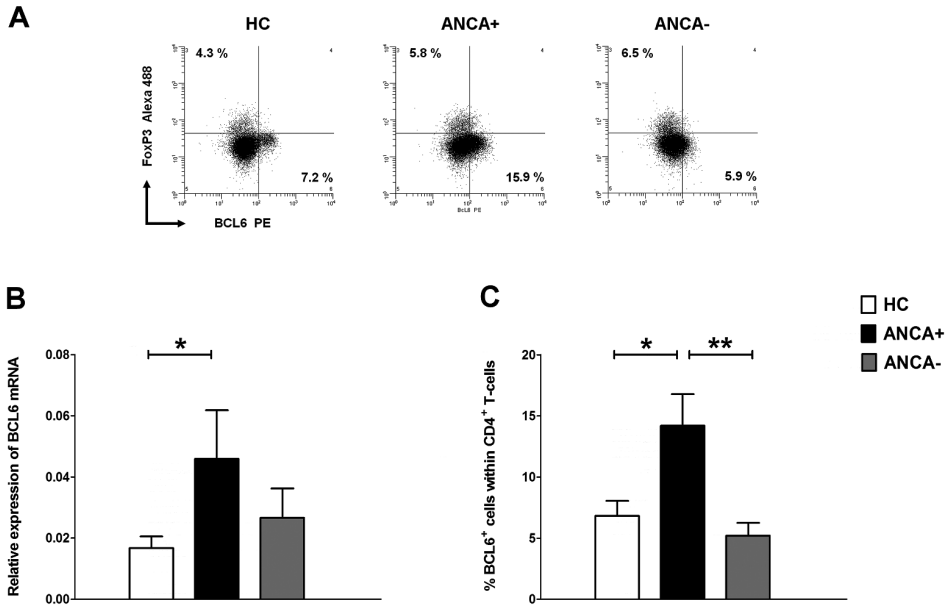


Figure 3. Expression of transcription factors BCL-6 and FoxP3. (A) Representative flow cytometry plots of BCL-6 versus FoxP3 expression in circulating $CD4^+$ T cells from an antineutrophil cytoplasmic antibody (ANCA)-negative (right plot) and an ANCA-positive (middle plot) patient with granulomatosis with polyangiitis (GPA) and an age- and sex-matched healthy control (HC) (left plot). Values in each gate represent the percentages of positive cells. (B) Relative mRNA expression of BCL-6 in leukocytes from ANCA-positive (n = 10) and ANCA-negative (n= 6) GPA patients, and age- and sex-matched HCs (n = 11) was analyzed by real-time RT-PCR and normalized to the housekeeping gene *GAPDH*. (C) Percentage of $BCL\text{-}6^+FoxP3^-$ cells in circulating $CD4^+$ T cells was determined in ANCA-positive (n = 11) and ANCA-negative (n = 10) GPA patients, and age- and sex-matched HCs (n = 10). Bars represent the mean values ±SD. *P*-values were calculated using the nonparametric Mann-Whitney U-test. * $P < 0.05$; ** $P < 0.005$.

Because in recent studies a new population of $FoxP3^+$ regulatory T cells has been described that shares features with T_{FH} cells by expressing the transcription factor BCL-6, we also evaluated whether the increase in $BCL\text{-}6^+$ T cells in GPA patients was a result of an increase in $FoxP3^+BCL\text{-}6^+$ T cells [36, 37]. This analysis showed that the increase in BCL-6 expression in GPA patients was restricted to T_{FH} cells and although a low percentage of $FoxP3^+BCL\text{-}6^+$ T cells was found (< 0.3%), no differences in these cell frequencies were observed between GPA patients and HCs (data not shown).

Proportions of IL-21-receptor expressing B cells do not differ between GPA patients and healthy controls

Since it is well known that IL-21 acts on B cells to support their expansion and antibody production [38-40], we conducted further analysis to compare the expression of IL-21R on B cells from GPA patients and HCs. No differences were seen in the percentages

of IL-21R$^+$ B cells either between ANCA-positive patients (n= 13) and ANCA-negative patients (n= 14) or between patients and HCs (n= 19) (Figure 4).

IL-21 induces IgG and ANCA production by B cells from GPA patients

To explore the interplay between IL-21 producing Th-cells and B cells in GPA patients, we investigated the effect of IL-21 on IgG antibody-production by B cells from GPA patients. Restricted numbers of patients and controls were enrolled in this analysis due to insufficient cell numbers. PBMCs from GPA patients were cultured *in vitro* in the presence or absence of exogenous IL-21 for 12 days and total IgG was measured in supernatants by ELISA. Because IL-21 promotes B cell differentiation by synergizing with BAFF [12, 13], we questioned whether the effect of IL-21 on IgG production could be augmented by adding BAFF to the culture. Of note, autologous T cells in our culture system act as a natural provider of CD40 ligation for B cells, as this ligation is required

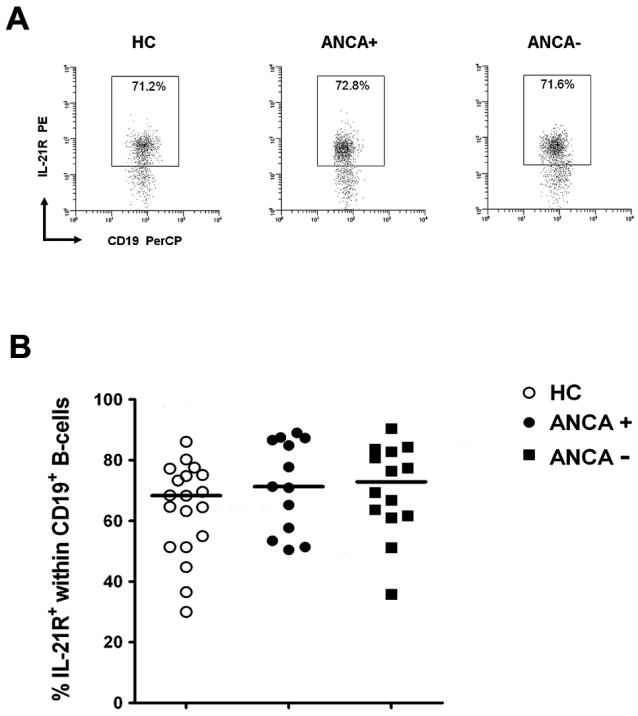


Figure 4. Comparison of IL-21R expressing B cells from patients with granulomatosis with polyangiitis (GPA) and healthy controls (HCs). (A) Representative flow cytometry plots of IL-21R expression on CD19$^+$ B cells from an ANCA-negative (right plot) and an ANCA-positive (middle plot) GPA patient, and an age- and sex-matched HC (left plot). Values in each gate represent the percentages of IL-21R$^+$ B cells. (B) The percentage of IL-21R$^+$ B cells was determined in peripheral blood from ANCA-positive (n = 13) and ANCA-negative (n= 14) GPA patients, and age- and sex-matched HCs (n = 19). Bars represent the mean values ± SD. *P*-values were calculated using the nonparametric Mann-Whitney U-test.

for B cell activation, isotype switching and memory development. As shown in figure 5, IL-21 significantly enhanced the production of IgG *in vitro* in stimulated PBMCs from both ANCA-positive (n= 7) and ANCA-negative (n= 6) GPA patients, whereas stimulation with BAFF alone did not result in increased IgG production. The combination of BAFF and IL-21 tended to increase IgG production more than IL-21 alone. Next, we assessed the effect of IL-21 plus BAFF on *in vitro* production of PR3-ANCA.

As shown in figure 5B, spontaneous PR3-ANCA production was observed in cultured PBMCs from ANCA-positive patients (n= 16) in comparison with cells from

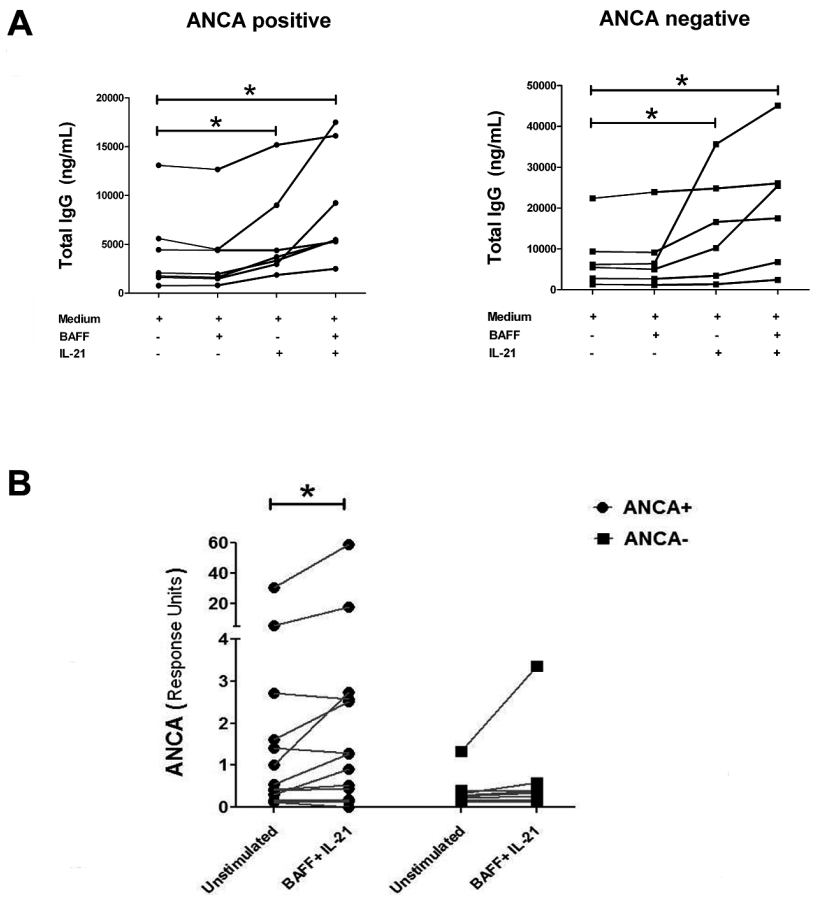


Figure 5. IL-21 induces *in vitro* immunoglobulin G (IgG) and PR3-ANCA production by B cells from patients with granulomatosis with polyangiitis (GPA). (**A**) PBMCs from ANCA-positive (n = 7) and ANCA-negative (n = 6) GPA patients were cultured in the presence of rhIL-21 and/or rhBAFF. Culture supernatants were collected after 12 days to measure total IgG by ELISA. (**B**) To assess the effect of IL-21 on *in vitro* ANCA-production, PBMCs from ANCA-positive (n = 16) and ANCA-negative (n = 12) GPA patients were stimulated in the presence of IL-21/BAFF. After 12 days, PR3-ANCA levels were determined by Phadia ImmunoCAP® 250 analyzer. *P*-values were calculated using the Wilcoxon matched pairs test. * $P < 0.05$.

ANCA-negative patients (n= 12). Importantly, IL-21 induces a significant enhancement in PR3-ANCA production in PBMCs isolated from ANCA-positive patients in comparison with ANCA-negative patients. So it is conceivable that autoreactive B cells were enriched in the peripheral blood of ANCA-positive patients.

DISCUSSION

In the present study, we demonstrate an increase in the percentage of circulating IL-21-producing Th-cells in GPA patients. We found that elevated frequencies of IL-21-producing Th-cells were restricted to ANCA-positive GPA patients and that these cells were distinct from Th17 cells. We also confirmed that IL-21 can enhance the production of IgG and ANCA *in vitro*.

Over the past few years, Th17 cells have challenged the classical Th1/Th2 paradigm, and have been implicated in a growing number of autoimmune and inflammatory diseases [41]. Recently, a distinct Th-cell subset termed T_{FH} and characterized by elevated expression levels of multiple surface proteins and BCL-6 as well as enhanced IL-21 secretion, has been identified as true helper cells for antibody responses. We and others have previously demonstrated that circulating Th17 cells are significantly increased in GPA patients even during quiescent disease [10, 11]. However, data are lacking to support a role of IL-21-producing Th-cells in GPA. Since Th17 cells also produce IL-21, we investigated whether Th17 cells in GPA are a source of IL-21. Strikingly, the majority of circulating CD4+ T cells that produced IL-21 were distinct from Th17 cells, indicating that other Th-cell subsets such as T_{FH} cells are the source of this cytokine. Importantly, the expansion of T_{FH} cells in GPA patients was confirmed by increased BCL-6 expression. To the best of our knowledge, this is the first report demonstrating an increase in the frequency of circulating IL-21-producing Th-cells in GPA suggesting that T_{FH} cell-derived IL-21 may contribute to disease pathogenesis via stimulation of (auto)antibody production.

T_{FH} cells are considered to be the major source of IL-21 and seem to be an important subset for adaptive immune responses, although there are conflicting reports on their mode of action *in vivo*. It has been demonstrated that IL-21-producing Th-cells induce Th17 development and proliferation [32, 33], which has been shown to promote germinal center (GC) formation in a BXD2 mouse model of autoimmunity [42]. In agreement with these findings, we demonstrate a significant positive relationship between $IL\text{-}17^{+}IL\text{-}21^{-}$ Th-cells and $IL\text{-}17^{-}IL\text{-}21^{+}$ Th-cells in peripheral blood of GPA patients. It seems likely that increased Th17 cells in GPA patients are the result of an enhanced T_{FH} response, which in turn may participate in granuloma formation and vascular damage. The role of IL-21 in vasculitis was previously suggested by Chen and coworkers [43]. In their study, mice deficient in interferon regulatory factor 4, a protein that inhibits IL-17A production, rapidly developed large-vessel vasculitis and showed increased IL-21 synthesis in addition to increased IL-17A production [43]. Moreover, a role of IL-21 in recruitment of Th17 cells to inflamed tissues has been reported by Caruso and coworkers [44] by showing that IL-21 induces gut

epithelial cells to secrete macrophage inflammatory protein 3α (MIP-3α), a chemokine that mediates Th17 cell homing to the skin, joints, and mucosal tissues. Given that endothelial cells are known to produce MIP-3α, it is possible that IL-21 in GPA patients enhances the migration and accumulation of Th17 cells into the vascular wall resulting in inflammation. Besides, IL-21 was shown to enhance granzyme B expression [45] and increase perforin-mediated cytotoxicity by human CD8+ T cells [46] and natural killer cells [47]. It is therefore conceivable that IL-21 can contribute to vessel injury and disease progression in GPA patients. This is an area worth of further investigation.

In contrast to the pro-inflammatory role of T_{FH} cells, recent studies have identified a distinctive population of T_{FH} cells that displays a regulatory function and suppresses the differentiation of GC B cells in follicles *in vivo*. This subset was termed follicular regulatory T cells (T_{FR}), which express the regulatory transcription factor *FoxP3* in addition to their specific lineage transcription factor BCL-6 [36, 37]. As circulating *FoxP3*$^+$ T cells are increased in GPA patients [7], it is conceivable that the observed increase in T_{FH} cells in patients is due to an increase in T_{FR} cells that co-express *FoxP3* and BCL-6. We have investigated this possibility but found that the increase in circulating T_{FH} cells in GPA patients cannot be explained by increase in T_{FR} cells (data not shown).

In our study, increased frequencies of T_{FH} cells were observed in patients who were ANCA-positive at the time of inclusion. This suggests the involvement of IL-21 in the process of autoantibody production in GPA. These data are in line with previous reports showing that T_{FH} cells act directly on B cells through the IL-21/IL-21R pathway, and that IL-21 is a potent inducer of class-switch recombination and plasma cell differentiation [39, 48, 49]. The expression of IL-21R on B cells from ANCA-positive and ANCA-negative GPA patients was comparable, which suggests that both patient populations have the same ability to respond to IL-21. However, *in vitro* stimulation with IL-21 enhanced the production of ANCA in cell cultures from ANCA-positive patients only, although enhanced total IgG production was observed in both patient groups. So it is conceivable that autoreactive B cells were enriched in the peripheral blood of ANCA-positive patients. This might be clinically relevant as well because ANCA positive patients are at increased risk for disease relapse [50, 51].

In this study, patients were evaluated for the distribution of T_{FH} cells during remission. We have previously shown that activated T cells are present at the time of clinically quiescent disease [9, 10]. Furthermore, during active disease effector T cells appear to migrate towards inflamed tissue [52]. Therefore, in order to study dysbalance of T cells in GPA patients using peripheral blood samples, we selected patients without or with low dosages of immunosuppressive medication and at the time of clinically quiescent disease.

CONCLUSIONS

In conclusion, the data presented here demonstrate a prominent increase of circulating T_{FH} cells in ANCA-positive GPA patients. The key cytokine of these T_{FH} cells, that is

IL-21, contributes to the production of ANCA autoantibodies *in vitro*. These data support the notion that T_{FH} cells are associated with the pathogenic process in GPA patients and may constitute a novel target for therapeutic intervention.

ACKNOWLEDGMENTS

We are grateful to the patients and healthy donors for their co-operation and participation in this study. The research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 261382, and from the Groningen University Institute for Drug Exploration (GUIDE).

REFERENCES

1. Fauci AS, Haynes BF, Katz P, Wolff SM: **Wegener's granulomatosis: prospective clinical and therapeutic experience with 85 patients for 21 years**. *Ann Intern Med* 1983, **98**:76-85.
2. van der Woude FJ, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, van der GM, van der Hem GK, The TH: **Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis**. *Lancet* 1985, **1**:425-429.
3. Niles JL, McCluskey RT, Ahmad MF, Arnaout MA: **Wegener's granulomatosis autoantigen is a novel neutrophil serine proteinase**. *Blood* 1989, **74**:1888-1893.
4. Brouwer E, Tervaert JW, Horst G, Huitema MG, van der GM, Limburg PC, Kallenberg CG: **Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis and clinically related disorders**. *Clin Exp Immunol* 1991, **83**:379-386.
5. Komocsi A, Lamprecht P, Csernok E, Mueller A, Holl-Ulrich K, Seitzer U, Moosig F, Schnabel A, Gross WL: **Peripheral blood and granuloma CD4(+)CD28(-) T cells are a major source of interferon-gamma and tumor necrosis factor-alpha in Wegener's granulomatosis**. *Am J Pathol* 2002, **160**:1717-1724.
6. Lamprecht P, Moosig F, Csernok E, Seitzer U, Schnabel A, Mueller A, Gross WL: **CD28 negative T cells are enriched in granulomatous lesions of the respiratory tract in Wegener's granulomatosis**. *Thorax* 2001, **56**:751-757.
7. Abdulahad WH, Stegeman CA, van der Geld YM, Doornbos-van der MB, Limburg PC, Kallenberg CG: **Functional defect of circulating regulatory CD4+ T cells in patients with Wegener's granulomatosis in remission**. *Arthritis Rheum* 2007, **56**:2080-2091.
8. Morgan MD, Day CJ, Piper KP, Khan N, Harper L, Moss PA, Savage CO: **Patients with Wegener's granulomatosis demonstrate a relative deficiency and functional impairment of T-regulatory cells**. *Immunology* 2010, **130**:64-73.
9. Abdulahad WH, van der Geld YM, Stegeman CA, Kallenberg CG: **Persistent expansion of CD4+ effector memory T cells in Wegener's granulomatosis**. *Kidney Int* 2006, **70**:938-947.
10. Abdulahad WH, Stegeman CA, Limburg PC, Kallenberg CG: **Skewed distribution of Th17 lymphocytes in patients with Wegener's granulomatosis in remission**. *Arthritis Rheum* 2008, **58**:2196-2205.
11. Nogueira E, Hamour S, Sawant D, Henderson S, Mansfield N, Chavele KM, Pusey CD, Salama AD: **Serum IL-17 and IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis**. *Nephrol Dial Transplant* 2010, **25**:2209-2217.
12. Ettinger R, Sims GP, Robbins R, Withers D, Fischer RT, Grammer AC, Kuchen S, Lipsky PE: **IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells**. *J Immunol* 2007, **178**:2872-2882.
13. Karnell JL, Ettinger R: **The Interplay of IL-21 and BAFF in the Formation and Maintenance of Human B Cell Memory**. *Front Immunol* 2012, **3**:2.
14. Young DA, Hegen M, Ma HL, Whitters MJ, Albert LM, Lowe L, Senices M, Wu PW, Sibley B, Leathurby Y, Brown TP, Nickerson-Nutter C, Keith JC, Jr., Collins M: **Blockade of the interleukin-21/interleukin-21 receptor pathway ameliorates disease in animal models of rheumatoid arthritis**. *Arthritis Rheum* 2007, **56**:1152-1163.
15. Herber D, Brown TP, Liang S, Young DA, Collins M, Dunussi-Joannopoulos K: **IL-21 has a pathogenic role in a lupus-prone mouse model and its blockade with IL-21R. Fc reduces disease progression**. *J Immunol* 2007, **178**:3822-3830.
16. Fina D, Sarra M, Fantini MC, Rizzo A, Caruso R, Caprioli F, Stolfi C, Cardolini I, Dottori M, Boirivant M, Pallone F, Macdonald TT, Monteleone G: **Regulation of gut inflammation and th17 cell response by interleukin-21**. *Gastroenterology* 2008, **134**:1038-1048.
17. Spolski R, Kashyap M, Robinson C, Yu Z, Leonard WJ: **IL-21 signaling is critical for the development of type I diabetes in the NOD mouse**. *Proc Natl Acad Sci U S A* 2008, **105**:14028-14033.
18. Sawalha AH, Kaufman KM, Kelly JA, Adler AJ, Aberle T, Kilpatrick J, Wakeland EK, Li QZ, Wandstrat AE, Karp DR, James JA, Merrill JT, Lipsky P, Harley JB: **Genetic association**

of interleukin-21 polymorphisms with systemic lupus erythematosus. *Ann Rheum Dis* 2008, **67**:458-461.

19. Festen EA, Goyette P, Scott R, Annese V, Zhernakova A, Lian J, Lefebvre C, Brant SR, Cho JH, Silverberg MS, Taylor KD, de Jong DJ, Stokkers PC, Mcgovern D, Palmieri O, Achkar JP, Xavier RJ, Daly MJ, Duerr RH, Wijmenga C, Weersma RK, Rioux JD: **Genetic variants in the region harbouring IL2/IL21 associated with ulcerative colitis**. *Gut* 2009, **58**:799-804.
20. Liu Y, Helms C, Liao W, Zaba LC, Duan S, Gardner J, Wise C, Miner A, Malloy MJ, Pullinger CR, Kane JP, Saccone S, Worthington J, Bruce I, Kwok PY, Menter A, Krueger J, Barton A, Saccone NL, Bowcock AM: **A genome-wide association study of psoriasis and psoriatic arthritis identifies new disease loci**. *PLoS Genet* 2008, **4**:e1000041.
21. Craft JE: **Follicular helper T cells in immunity and systemic autoimmunity**. *Nat Rev Rheumatol* 2012, **8**:337-347.
22. Dolff S, Abdulahad WH, Westra J, Doornbos-van der MB, Limburg PC, Kallenberg CG, Bijl M: **Increase in IL-21 producing T-cells in patients with systemic lupus erythematosus**. *Arthritis Res Ther* 2011, **13**:R157.
23. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, Manku H, Vyse TJ, Roncador G, Huttley GA, Goodnow CC, Vinuesa CG, Cook MC: **Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus**. *Arthritis Rheum* 2010, **62**:234-244.
24. Ma J, Zhu C, Ma B, Tian J, Baidoo SE, Mao C, Wu W, Chen J, Tong J, Yang M, Jiao Z, Xu H, Lu L, Wang S: **Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis**. *Clin Dev Immunol* 2012, **2012**:827480.
25. Jennette JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, Hagen EC, Hoffman GS, Hunder GG, Kallenberg CG, .: **Nomenclature of systemic vasculitides. Proposal of an international consensus conference**. *Arthritis Rheum* 1994, **37**:187-192.
26. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, Calabrese LH, Fries JF, Lie JT, Lightfoot RW, Jr., .: **The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis**. *Arthritis Rheum* 1990, **33**:1101-1107.
27. Luqmani RA, Bacon PA, Moots RJ, Janssen BA, Pall A, Emery P, Savage C, Adu D: **Birmingham Vasculitis Activity Score (BVAS) in systemic necrotizing vasculitis**. *QJM* 1994, **87**:671-678.
28. Tervaert JW, Mulder L, Stegeman C, Elema J, Huitema M, The H, Kallenberg C: **Occurrence of autoantibodies to human leucocyte elastase in Wegener's granulomatosis and other inflammatory disorders**. *Ann Rheum Dis* 1993, **52**:115-120.
29. Tervaert JW, Goldschmeding R, Elema JD, van der GM, Huitema MG, van der Hem GK, The TH, von dem Borne AE, Kallenberg CG: **Autoantibodies against myeloid lysosomal enzymes in crescentic glomerulonephritis**. *Kidney Int* 1990, **37**:799-806.
30. Savige J, Dimech W, Fritzler M, Goeken J, Hagen EC, Jennette JC, McEvoy R, Pusey C, Pollock W, Trevisin M, Wiik A, Wong R: **Addendum to the International Consensus Statement on testing and reporting of antineutrophil cytoplasmic antibodies. Quality control guidelines, comments, and recommendations for testing in other autoimmune diseases**. *Am J Clin Pathol* 2003, **120**:312-318.
31. Tadema H, Abdulahad WH, Lepse N, Stegeman CA, Kallenberg CG, Heeringa P: **Bacterial DNA motifs trigger ANCA production in ANCA-associated vasculitis in remission**. *Rheumatology (Oxford)* 2011, **50**:689-696.
32. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK: **IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells**. *Nature* 2007, **448**:484-487.
33. Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, Schluns K, Tian Q, Watowich SS, Jetten AM, Dong C: **Essential autocrine regulation by IL-21 in the generation of inflammatory T cells**. *Nature* 2007, **448**:480-483.
34. Yu D, Rao S, Tsai LM, Lee SK, He Y, Sutcliffe EL, Srivastava M, Linterman M, Zheng L, Simpson N, Ellyard JI, Parish IA, Ma CS, Li QJ, Parish CR, Mackay CR, Vinuesa CG: **The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment**. *Immunity* 2009, **31**:457-468.

35. Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, Wang YH, Dong C: **Bcl6 mediates the development of T follicular helper cells**. *Science* 2009, **325**:1001-1005.
36. Chung Y, Tanaka S, Chu F, Nurieva RI, Martinez GJ, Rawal S, Wang YH, Lim H, Reynolds JM, Zhou XH, Fan HM, Liu ZM, Neelapu SS, Dong C: **Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions**. *Nat Med* 2011, **17**:983-988.
37. Linterman MA, Pierson W, Lee SK, Kallies A, Kawamoto S, Rayner TF, Srivastava M, Divekar DP, Beaton L, Hogan JJ, Fagarasan S, Liston A, Smith KG, Vinuesa CG: **Foxp3+ follicular regulatory T cells control the germinal center response**. *Nat Med* 2011, **17**:975-982.
38. Pene J, Gauchat JF, Lecart S, Drouet E, Guglielmi P, Boulay V, Delwail A, Foster D, Lecron JC, Yssel H: **Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells**. *J Immunol* 2004, **172**:5154-5157.
39. Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, Leonard WJ, Lipsky PE: **IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells**. *J Immunol* 2005, **175**:7867-7879.
40. Kuchen S, Robbins R, Sims GP, Sheng C, Phillips TM, Lipsky PE, Ettinger R: **Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration**. *J Immunol* 2007, **179**:5886-5896.
41. Waite JC, Skokos D: **Th17 response and inflammatory autoimmune diseases**. *Int J Inflam* 2012, **2012**:819467.
42. Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, Yi J, Guentert T, Tousson A, Stanus AL, Le TV, Lorenz RG, Xu H, Kolls JK, Carter RH, Chaplin DD, Williams RW, Mountz JD: **Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice**. *Nat Immunol* 2008, **9**:166-175.
43. Chen Q, Yang W, Gupta S, Biswas P, Smith P, Bhagat G, Pernis AB: **IRF-4-binding protein inhibits interleukin-17 and interleukin-21 production by controlling the activity of IRF-4 transcription factor**. *Immunity* 2008, **29**:899-911.
44. Caruso R, Fina D, Peluso I, Stolfi C, Fantini MC, Gioia V, Caprioli F, Del Vecchio BG, Paoluzi OA, Macdonald TT, Pallone F, Monteleone G: **A functional role for interleukin-21 in promoting the synthesis of the T-cell chemoattractant, MIP-3alpha, by gut epithelial cells**. *Gastroenterology* 2007, **132**:166-175.
45. Liu Y, Yang B, Ma J, Wang H, Huang F, Zhang J, Chen H, Wu C: **Interleukin-21 induces the differentiation of human Tc22 cells via phosphorylation of signal transducers and activators of transcription**. *Immunology* 2011, **132**:540-548.
46. Ebert EC: **Interleukin 21 up-regulates perforin-mediated cytotoxic activity of human intra-epithelial lymphocytes**. *Immunology* 2009, **127**:206-215.
47. Liu Z, Yang L, Cui Y, Wang X, Guo C, Huang Z, Kan Q, Liu Z, Liu Y: **Il-21 enhances NK cell activation and cytolytic activity and induces Th17 cell differentiation in inflammatory bowel disease**. *Inflamm Bowel Dis* 2009, **15**:1133-1144.
48. Ozaki K, Spolski R, Ettinger R, Kim HP, Wang G, Qi CF, Hwu P, Shaffer DJ, Akilesh S, Roopenian DC, Morse HC, III, Lipsky PE, Leonard WJ: **Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6**. *J Immunol* 2004, **173**:5361-5371.
49. Linterman MA, Beaton L, Yu D, Ramiscal RR, Srivastava M, Hogan JJ, Verma NK, Smyth MJ, Rigby RJ, Vinuesa CG: **IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses**. *J Exp Med* 2010, **207**:353-363.
50. Slot MC, Tervaert JW, Boomsma MM, Stegeman CA: **Positive classic antineutrophil cytoplasmic antibody (C-ANCA) titer at switch to azathioprine therapy associated with relapse in proteinase 3-related vasculitis**. *Arthritis Rheum* 2004, **51**:269-273.
51. Pierrot-Deseilligny DC, Pouchot J, Pagnoux C, Coste J, Guillevin L: **Predictors at diagnosis of a first Wegener's granulomatosis relapse after obtaining complete remission**. *Rheumatology (Oxford)* 2010, **49**:2181-2190.
52. Abdulahad WH, Kallenberg CG, Limburg PC, Stegeman CA: **Urinary CD4+ effector memory T cells reflect renal disease activity in antineutrophil cytoplasmic antibody-associated vasculitis**. *Arthritis Rheum* 2009, **60**:2830-2838.

CHAPTER 4

TLR-9 ACTIVATION ENHANCES B CELL ACTIVATING FACTOR AND IL-21 INDUCED ANTI-PROTEINASE 3 AUTOANTIBODY PRODUCTION *IN VITRO*

Nikola Lepse, Judith Land, Abraham Rutgers,
Cees G.M. Kallenberg, Coen A. Stegeman,
Wayel H. Abdulahad and Peter Heeringa

MANUSCRIPT IN PREPARATION

ABSTRACT

Introduction

Granulomatosis with polyangiitis (GPA) is a relapsing small-vessel vasculitis characterized by the presence of circulating anti-neutrophil cytoplasmic antibodies (ANCA) against proteinase 3 (PR3). The mechanisms that trigger PR3-ANCA production are unknown. The aim of this study was to determine whether endogenous (BAFF, IL-21) and exogenous factors (TLR9 ligand CpG-ODN) synergize in stimulating PR3-ANCA production in GPA patients.

Methods

Peripheral blood mononuclear cells (PBMC) from GPA patients and healthy controls (HC) were cultured in the presence of BAFF and IL-21, with or without CpG-ODN for 12 days. ANCA production in culture supernatants was quantified by Phadia EliA. Phenotypic characterization and the influence of CpG-ODN treatment on IL-21 receptor (IL-21R), TACI and BAFF-R expression on B cell subsets was analysed by flow cytometry.

Results

Stimulation with BAFF and IL-21 significantly increased ANCA production in patient samples which could be further augmented by addition of CpG-ODN. PBMC stimulation with CpG-ODN for 24 h increased the percentage of IL-21R+ and TACI+ B cells, but did not affect BAFF-R expression. GPA patients had an increased percentage of circulating IL-21R+ memory B cells and a decreased percentage of TACI+ memory B cells when compared to HC. Patients had decreased BAFF-R expression levels on B cells, which were inversely correlated with BAFF levels in plasma.

Conclusions

Our data demonstrate that endogenous and exogenous factors can synergize to promote PR3-ANCA production. Mechanistically, CpG-ODN up-regulated IL-21R and TACI expression on B cells, possibly sensitizing these cells for IL-21 and BAFF mediated signals. Agents inhibiting BAFF and IL-21 signalling pathways are potential targets for intervention in GPA patients.

INTRODUCTION

Granulomatosis with polyangiitis (GPA) is characterized by necrotizing inflammation of the small- to medium-sized blood vessels, frequently affecting the respiratory tract and kidneys [1]. A hallmark of GPA is the presence of circulating anti-neutrophil cytoplasmic antibodies (ANCA), which are mainly directed against proteinase 3 (PR3) [1, 2]. In patients, a rising serum titer of PR3-ANCA often precedes a disease relapse supporting the notion that autoantibodies are involved in disease pathogenesis. The pathogenic potential of PR3-ANCA has been studied extensively both *in vitro* and *in vivo*. Neutrophil and monocyte stimulation *in vitro* with PR3-ANCA induces cell activation, resulting in the generation of reactive oxygen species (ROS), neutrophil degranulation, and pro-inflammatory cytokine production [3-5]. More recently, experiments in humanized mice demonstrated that PR3-ANCA can induce acute vascular injury providing further support for a direct pathogenic role of these autoantibodies [6].

Although the pathogenic potential of PR3-ANCA is well established, the mechanisms that trigger PR3-ANCA production and disease relapse are less clear. Based on clinical observations, infections have been proposed to play an important role in GPA pathogenesis. In GPA patients, chronic nasal carriage of *Staphylococcus aureus* (*S. aureus)* is strongly linked to relapsing disease, and maintenance treatment with the antibiotic co-trimoxazole can prevent relapses [7, 8]. The association with infections is further supported by *in vitro* studies demonstrating that B cell stimulation with Toll-like receptor (TLR) 9 ligand CpG-ODN can boost the production of PR3-ANCA [9, 10].

Because ANCA predominantly belong to IgG1 and IgG4 subclasses, this suggests involvement of an antigen-driven T cell-dependent immune response in the generation of ANCA-specific B cells [11, 12]. In particular, a specific T helper cell subset characterized by production of IL-21 provides signals to B cells supporting generation of high-affinity clones and differentiation to plasma cells [13]. We have recently reported increased frequencies of IL-21-producing CD4+ T helper cells in the circulation of GPA patients in remission [14]. IL-21 signaling is context dependent and co-stimulatory signals have a critical influence on the resultant biological effect. In combination with CD40 signaling IL-21 stimulates naive B cell proliferation and differentiation, whereas in conjunction with B cell activating factor (BAFF) IL-21 can stimulate plasma cell formation and antibody production by antigen-experienced B cells [15, 16]. BAFF is a cytokine crucial for B cell survival, proliferation and differentiation, and over-expression of BAFF has been strongly associated with the development of systemic autoimmunity [17]. Increased BAFF levels have been found in patients with various autoimmune disorders, including systemic lupus erythematosus (SLE) and Sjogren's syndrome (SS), as well as GPA [18-21].

The observation that in GPA the proportion of IL-21+ T helper cells is increased and BAFF levels are elevated even during clinical remission, suggests that in these patients an environment exists that facilitates survival and activation of autoreactive B cells. As the relapsing nature of GPA has been associated with infections, we hypothesized that infectious agents can synergize with BAFF and IL-21 present in the endogenous

environment to stimulate autoantibody production in GPA patients. To test this hypothesis, we investigated the effect of IL-21, BAFF and the TLR9 ligand CpG-ODN on PR3-ANCA production, B cell proliferation and plasma cell formation. Furthermore, we evaluated the expression of receptors for IL-21 and BAFF on circulating B cells and studied the effect of CpG-ODN stimulation on the expression of these receptors in GPA patients and HC.

PATIENTS AND METHODS

Study population

The characteristics of patients and HC included in the study are described in table 1. Two cohorts of GPA patients and HC were included in the study. Cohort 1 was used to establish the findings of *in vitro* ANCA production assay. Cohort 2 was used to characterize the expression of IL-21R, BAFF-R, and TACI on B cell subsets. The diagnosis GPA was based on the definitions outlined in the Chapel Hill Consensus Conference and patients fulfilled the classification criteria of the American College of Rheumatology (ACR) [22, 23]. Samples were obtained in compliance with the Declaration of Helsinki. All subjects provided informed consent, and the study

Table 1. Characteristics of the study population

Characteristics		HC	GPA Remission	GPA Active
Cohort 1				
n (% of males)		15 (67%)	15 (58%)	6 (64%)
Age, mean (range)		56.5 (45-64)	57.8 (32-77)	63.3 (42-78)
PR3-ANCA titer, median (range)		NA	1:80 (1:20-1:640)	1:240 (1:80-1:640)
Treatment	AZA (100 mg/day)	NA	2	1
	Pred (5 – 10 mg/day)	NA	3	2
	AZA (100 mg/day) / Pred (5 mg/day)	NA	1	0
	No immunosuppressive therapy	NA	9	3
BVAS, median (range)		NA	0	18 (10 – 20)
Cohort 2				
n (% of males)		15 (60%)	21 (38%)	0 (0%)
Age, mean (range)		56.3 (45-65)	60.1 (32-84)	NA
PR3-ANCA titer, median (range)		NA	1:80 (0-1:640)	NA
Treatment	AZA (100 mg/day)	NA	6	NA
	Pred (5 – 10 mg/day)	NA	3	NA
	AZA (100 mg/day) / Pred (5 mg/day)	NA	2	NA
	MMF / Pred	NA	1	NA
	No immunosuppressive therapy	NA	9	NA
BVAS, median (range)		NA	0	NA

ANCA, anti-neutrophil cytoplasmic autoantibodies; AZA, azathioprine; BVAS, Birmingham Vasculitis Activity Score; GPA, granulomatosis with polyangiitis; HC, healthy controls; MMF, mycophenolate mofetil; NA, not applicable; PR3, proteinase 3; Pred, prednisolone.

was approved by the Medical Ethics Committee of the University Medical Center Groningen, University of Groningen (NL).

Peripheral blood mononuclear cell (PBMC) isolation and culture

Heparinized blood was collected from patients and HC, diluted 1:1 in PBS (pH 7.4), overlaid on Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged for 20 minutes at 600 x g. PBMC were collected and washed twice with PBS. PBMC were re-suspended in culture medium (RPMI 1640 (Lonza, Basel, Switzerland), supplemented with 50 µg/mL gentamicin (GIBCO, Life Technologies, Grand Island, NY, USA) and 10% fetal calf serum (FCS; Lonza)) at a concentration of $1x10^6$ cells/mL. Cells were cultured in a 24-well Costar plate (Corning Inc., Corning, NY, USA) in the presence of 100 ng/mL BAFF (PeproTech Inc., Rocky Hill, NJ, USA), 100 ng/mL IL-21 (Immunotools, Friesoythe, Germany) with or without 3.2 µg/mL CpG-ODN 2006 (Hycult Biotech, Uden, the Netherlands) at 37 °C with 5% CO_2. After 12 days of stimulation, the culture supernatants were collected and stored at -20 °C until further use.Quantification of PR3-ANCA

The level of PR3-ANCA in culture supernatants and patient serum samples was measured using Phadia ImmunoCAP® 250 analyzer using EliA PR3^S (Thermo Fisher Scientific, Waltham, MA, USA) and the levels of PR3-ANCA IgG were expressed in response units (RU). To determine the number of patients that produced significant levels of PR3-ANCA *in vitro*, the signal measured in HC samples was used to calculate a cut-off value (cut off = mean level in HC + 3*SD). Patients that were above the threshold were considered to produce a significant amount of PR3-ANCA *in vitro*.

Enzyme-linked immunosorbent assay (ELISA) for measurement of IgG

IgG in PBMC culture supernatants was quantified using an in-house ELISA. Briefly, a 96 Well Clear Flat Bottom Polystyrene High Bind Microplate (Corning Inc.) was coated with 1.3 µg/mL goat anti-human IgG F(ab')2 fragments (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in PBS. Plates were washed with washing buffer (0.025M Tris-HCl, 0.15M NaCl, 0.05% Tween-20) and blocked for 1 h with blocking buffer (PBS with 0.05% Tween-20 and 2% bovine serum albumin (BSA)). The supernatants were diluted in incubation buffer (PBS with 0.05% Tween-20 and 1% BSA). Purified human IgG (Siemens, Marburg, Germany) with a known concentration was used as a standard sample. The bound IgG was detected with mouse-anti-human-IgG-HRP (SouthernBiotech, Birmingham, AL, USA). Tetramethylbenzidine dihydrochloride (TMB; Sigma-Aldrich, St Louis, MO, USA) was used as substrate and optical density was read at 450 nm using an Emax microplate reader (Molecular Devices, Silicon Valley, CA, USA).

B cell proliferation assay

Freshly isolated PBMC were stained for 10 minutes with 500 ng/mL of carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Life Technologies, Grand Island, NY, USA). In order to quench the staining, cells were washed twice with culture medium. Then cells were suspended at a concentration of 10xE6 cells/mL, seeded in a 24

well Costar plate, and treated with 3.2 µg/mL CpG-ODN, 100 ng/mL BAFF, 100 ng/mL IL-21, combinations of the stimuli, or were left without stimulation. After 4 days of incubation, cells were harvested, washed with PBS + 0.3% heparin (LEO Pharma BV, Amsterdam, the Netherlands), re-suspended in 100 µL PBS + 0.3% heparin, and labeled with anti-human CD19-eFluor-450 (eBioscience, San Diego, CA, USA; clone HIB19) and anti-human CD22-PE-Cy5 (BD Biosciences, San Jose, CA, USA; clone HIB22). Subsequently cells were treated with FACS Lysing Solution (BD Biosciences), washed again and measured with BD™ LSR-II flow cytometer (BD, Franklin Lakes, NJ, USA). The data was analyzed using FlowJo Analysis Software (Tree Star, Inc., Ashland, OR, USA). The percentage of proliferated B cells was determined based on the intensity of the CFSE staining and data is expressed as the percentage of B cells, which have undergone at least 1 round of cell division.

Plasma cell formation assay

Freshly isolated PBMC were suspended in culture medium at a concentration of 10xE6 cells/mL and treated with 3.2 µg/mL CpG-ODN, 100 ng/mL BAFF, 100 ng/mL IL-21, combinations of the stimuli, or were left without stimulation. After 7 days, cells were harvested, washed with PBS + 1% BSA, re-suspended in 100 µL PBS + 1% BSA, and labeled with anti-human CD19-eFluor-450, anti-human CD22-PE-Cy5, anti-human CD27-APC-eFluor-780 (eBioscience), and anti-human CD38-PE-Cy7 (eBioscience). Then cells were treated with FACS Lysing Solution (BD Biosciences), washed, and measured with BD™ LSR-II flow cytometer. The data was analyzed using Kaluza 1.2 Flow Analysis Software (Beckman Coulter, Brea, CA, USA) and cells with CD19+CD27^{high}CD38^{high} phenotype were considered plasma cells.

Characterization of IL-21R, BAFF-R and TACI expression of B cell subsets by flow cytometry

Freshly drawn EDTA blood was washed twice with PBS + 1% BSA to remove the plasma. After washing, cells were suspended in PBS + 1% BSA to the original volume, and 100 µL of the cell suspension was incubated with anti-human CD19-PerCP (BD Biosciences; clone 4G7), anti-human CD27-FITC (BD Biosciences; clone M-T271), anti-human CD38-APC (BD Biosciences; clone HB7), and either anti-human IL-21R-PE (BD Biosciences; clone 17A12), anti-human BAFF-R-PE (BioLegend, San Diego, CA, USA; clone 11C1), or anti-human TACI-PE (BioLegend; clone 1A1), or the corresponding PE-conjugated isotype control antibodies for 15 minutes in the dark. The red blood cells were lysed with FACS Lysing Solution (BD Biosciences). Cells were washed with PBS + 1% BSA and analyzed with a BD™ LSR-II flow cytometer. The data was analyzed using Kaluza 1.2 Flow Analysis Software.

Alternatively, freshly isolated PBMC were suspended to a concentration of 10xE6 cells/mL in culture medium. Part of the cells was directly used for staining to measure the baseline expression of IL-21R, BAFF-R and TACI. Cells were seeded in a 24-well Costar plate and stimulated with CpG-ODN (3.2 µg/mL) for 4 or 24 hours. At the

end of the experiment, cells were harvested and washed with PBS + 5% FCS. Then cells were suspended in 100 µL PBS + 5% FCS + 2 nM EDTA and stained for surface expression of IL-21R, BAFF-R and TACI using the staining protocol described above for the whole blood samples.

ELISA for quantification of BAFF levels in plasma

BAFF levels in plasma were measured using Human BAFF Quantikine ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The plasma sample was collected on the same day as the blood sample used for measuring IL-21R, TACI and BAFF-R expression by flow cytometry.

Statistical analysis

Data are presented as median values, unless stated otherwise. Data from HC and GPA patients was compared using unpaired *t* test or Mann-Whitney test for data with Gaussian and non-Gaussian distribution, respectively. For intra-individual comparison of more than two groups the Repeated Measures ANOVA was used if data was normally distributed and Friedman test was used if data had non-Gaussian distribution as determined by D'Agostino and Perason omnibus normality test. If a significant difference was found, further testing was done using a paired *t* test or Wilcoxon matched pairs test for data with Gaussian and non-Gaussian distribution, respectively. The significance of correlations was assessed using Spearman's rank correlation coefficient. P value < 0.05 was considered statistically significant. *P<0.05; **P<0.001; ***P<0.0001.

RESULTS

CpG-ODN, BAFF and IL-21 promote PR3-ANCA production *in vitro*

First, we evaluated the effect of BAFF, IL-21 and CpG-ODN on IgG production *in vitro*. Although treatment with CpG-ODN significantly increased IgG levels when compared to unstimulated cells, the combination of BAFF and IL-21 stimulated IgG production more potently than treatment with CpG-ODN alone (figure 1A). Furthermore, we found that the stimulatory effect of BAFF and IL-21 could be further enhanced by addition of CpG-ODN (figure 1A). As BAFF, IL-21 and CpG-ODN had a strong stimulatory effect on IgG production, we next tested the effect of BAFF and IL-21 whether in combination with CpG-ODN or not, on PR3-ANCA production *in vitro* (figure 1B). The combination of BAFF and IL-21 significantly increased PR3-ANCA production compared to unstimulated cells; this effect was significantly enhanced by addition of CpG-ODN (figure 1B). Eighteen out of 21 patients produced significant amounts of PR3-ANCA *in vitro* (figure 1D). To determine whether cells from patients and HC have similar capability to respond to the given stimuli, total IgG levels were measured in the supernatants. The amount of IgG produced by GPA patients and HC upon stimulation with CpG-ODN, BAFF and IL-21 was found to be comparable (figure 1C).

To investigate whether the observed *in vitro* ANCA production was related to PR3-ANCA serum level, we measured PR3-ANCA levels in serum samples from the corresponding patients collected at the time of cell isolation. A significant positive correlation was found between serum levels of PR3-ANCA and the amount of PR3-ANCA produced *in vitro* (figure 1E).

CpG-ODN induces B cell proliferation and plasma cell formation

Treatment with BAFF, IL-21 and CpG-ODN was a substantially better inducer of (auto)-antibody production than stimulation with BAFF and IL-21. CpG-ODN is known to promote formation of plasma blasts [24] and together with BAFF it has been reported to affect B cell activation, proliferation and immunoglobulin secretion [25, 26]. Therefore, we evaluated the effect of BAFF, IL-21 and CpG-ODN in comparison to BAFF and IL-21 on B cell proliferation and plasma cell formation (figure 2).

Stimulation with BAFF, IL-21 or the combination of both did not induce B cell proliferation when compared to unstimulated cells. In contrast, CpG-ODN alone significantly induced B cell proliferation, and the combined treatment with BAFF, IL-21 and CpG-ODN was not superior to stimulation with CpG-ODN alone, suggesting that the effect on B cell proliferation is mainly mediated by CpG-ODN (figure 2A).

As the combined treatment of CpG-ODN, BAFF and IL-21 could strongly stimulate IgG production *in vitro*, we also evaluated the effect of these stimuli on plasma cell formation. The combination of BAFF and IL-21 significantly promoted B cell differentiation towards plasma cells when compared to unstimulated cells. Also, treatment with CpG-ODN alone significantly promoted plasma cell formation, and the effect was comparable to that of CpG-ODN, BAFF, and IL-21 together, demonstrating that also plasma cell formation in this system is primarily driven by CpG-ODN (figure 2B).

CpG-ODN up-regulates IL-21R and TACI but not BAFF-R expression on B cells

As we observed that combined treatment with BAFF, IL-21 and CpG-ODN is a more potent inducer of PR3-ANCA production compared to BAFF and IL-21, we questioned whether stimulation with CpG-ODN can sensitize B cells to become more responsive to stimulation with IL-21 and BAFF. To this end, we evaluated the effect of treatment with CpG-ODN on the expression of the receptors for IL-21 and BAFF. IL-21 signals through IL-21R, which is mainly expressed on transitional and naïve B cells, and to a lesser extent on memory B cells [15]. BAFF is known to signal through 3 receptors, namely, BAFF receptor (BAFF-R), transmembrane activator and calcium modulator ligand interactor (TACI), and B cell maturation antigen (BCMA). As BCMA is mainly expressed on terminally differentiated plasma cells and is nearly absent on B cells [16, 27], we studied the expression of BAFF-R and TACI, which are abundantly expressed on B cells.

PBMC from HC and GPA patients were stimulated with CpG-ODN and expression of IL-21R, TACI and BAFF-R on B cells was evaluated by flow cytometry. Already after 4 hours of stimulation with CpG-ODN, a moderate increase in IL-21R+ B cells was

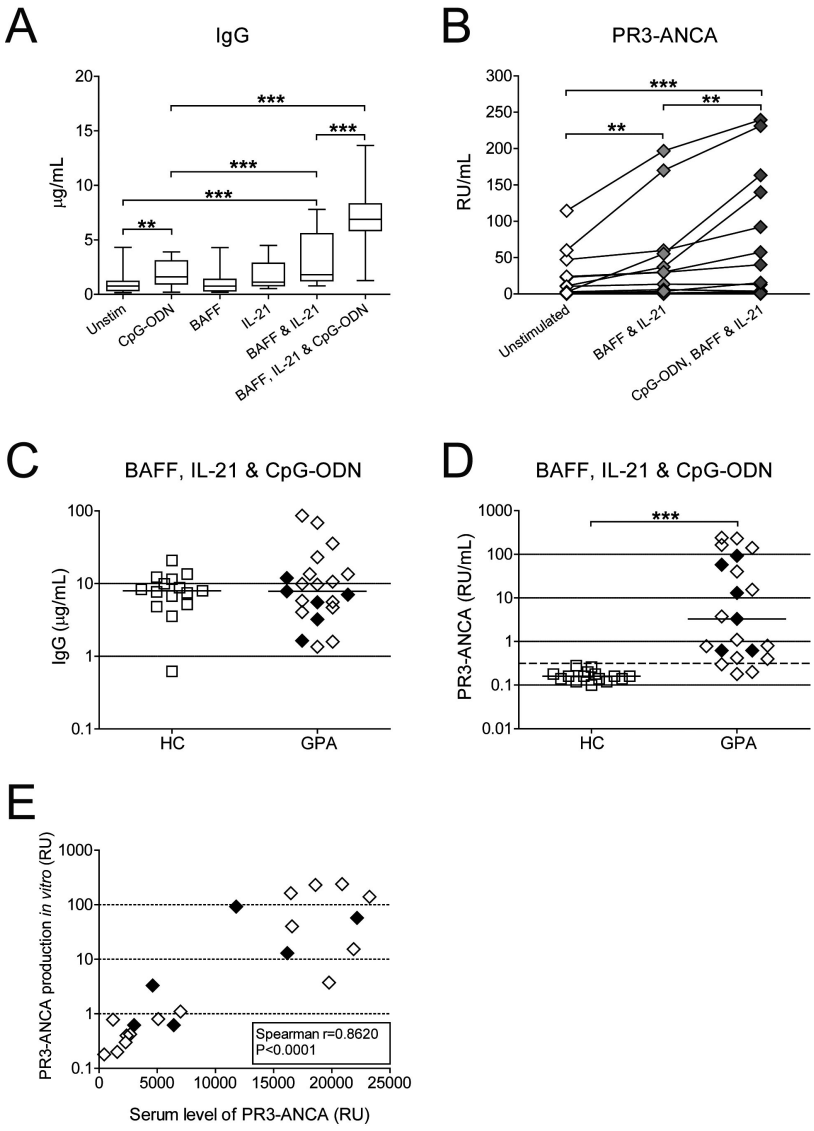


Figure 1. BAFF, IL-21 and CpG-ODN promote PR3-ANCA production *in vitro*. (A) The effect of CpG-ODN, BAFF and IL-21 on IgG production was initially tested in 12 GPA patients. As the combined treatment with BAFF/IL-21 and CpG-ODN/BAFF/IL-21 induced IgG production most potently, these stimuli were used in further experiments to stimulate PR3-ANCA production *in vitro*. (B) PBMC stimulation with BAFF and IL-21, or in combination with CpG-ODN significantly augmented PR3-ANCA production *in vitro*. Graph demonstrates data of 21 GPA patients (cohort 1). (C) Upon stimulation with BAFF, IL-21 and CpG-ODN, GPA patients and HC produced similar amounts of IgG. (D) Samples from HC (stimulated with BAFF, IL-21 and CpG-ODN) were used to calculate a cut-off level and patients above the cut-off were considered to produce significant amounts of PR3-ANCA *in vitro*. (E) The level of PR3-ANCA produced *in vitro* significantly correlated with the PR3-ANCA level in serum. (C-E) White diamonds represent GPA patients in clinical remission; black diamonds represent patients with active disease at the time of inclusion. **P<0.001; ***P<0.0001.

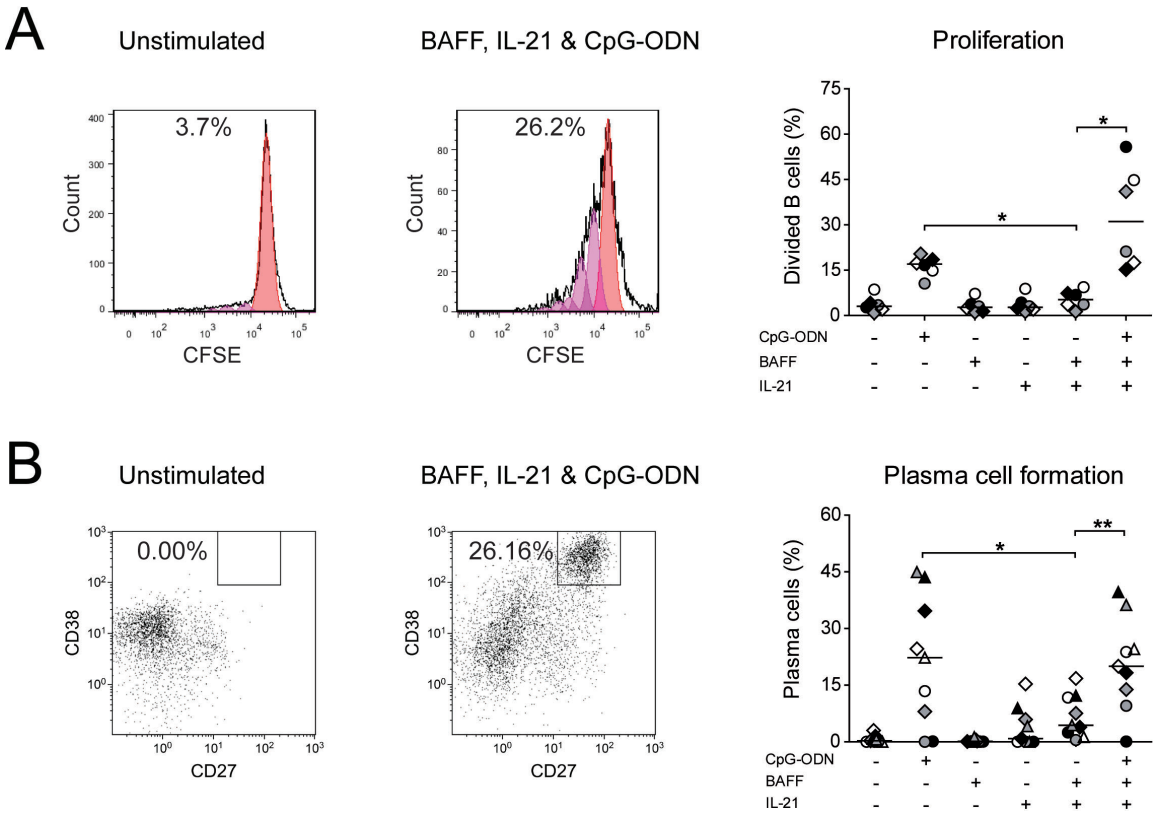


Figure 2. CpG-ODN mediates B cell proliferation and plasma cell formation. (**A**) Representative plots from 1 HC showing the effect of 4 day treatment with BAFF, IL-21 and CpG-ODN on B cell proliferation compared to unstimulated cells. The scatter plot shows data from 6 HC, and each symbol represents 1 donor. (**B**) Representative plots from 1 HC demonstrating plasma cell formation induced after 7 day treatment with BAFF, IL-21 and CpG-ODN. The graph shows data of 9 healthy individuals each represented with different symbols. (**A-B**) Paired samples were used for the statistical analysis; *P<0.05; **P<0.001.

observed when compared to baseline expression, and a further increase in IL-21R expression was observed after 24 hours (figures 3A and 3B). The IL-21R expression level, which was induced after 24 hour stimulation with CpG-ODN, was comparable between HC and GPA patients (Figure 3C).

Treatment with CpG-ODN also had a significant effect on TACI expression after 24 hour stimulation (figures 3A and 3B), when an increase in TACI expression was observed in all HC and 5 out of 6 GPA patients (figure 3C). The median percentage of TACI positive B cells after CpG-ODN treatment was not different between HC and patients.

In the majority of donors, all B cells were positive for BAFF-R, therefore its expression level was characterized based on mean fluorescence intensity. Unlike IL-21R and TACI, the expression of BAFF-R was not significantly affected by treatment with CpG-ODN (figure 3).

Divergent IL-21R and TACI expression on naive and memory B cells and decreased BAFF-R expression in GPA patients

We evaluated the expression of IL-21R, TACI and BAFF-R on total B cells as well as on naive and memory B cell subsets in 15 HC and 21 GPA patients. In accordance with previous findings [14], the median percentage of IL-21R+ B cells was comparable between HC and patients (71.6% and 73.5% in HC and GPA patients, respectively). However, when B cells were subdivided into naive and memory B cell populations, we found increased percentages of IL-21R+ memory B cells in patients when compared to HC (25.6% in HC, 38.6% in patients; P=0.0050). This pattern of skewing in receptor expression would favor antigen-experienced B cell stimulation by IL-21 (figure 4A).

Likewise, the median frequency of TACI+ B cells was not significantly different between HC and GPA patients (33.2% and 39.7%, respectively). When TACI expression was analyzed in naive and memory B cell subsets, GPA patients had an increased frequency of TACI-expressing naive B cells (12.5% in HC, 19.1% in GPA; P=0.0147) and a decreased percentage of TACI+ memory B cells when compared to HC (91.7% and 79.3%, respectively; P=0.0147) (figure 4B).

Finally, we assessed BAFF-R expression on B cells. Although overall BAFF-R expression level was highly variable in both HC and GPA patients (MFI range 14.4-76.5 in HC; 0.5-52.7 in GPA), GPA patients had significantly lower expression of BAFF-R on B cells when compared to HC (P=0.0113) (figure 4C). Previous studies have reported internalization of BAFF-R upon binding to its ligand and circulating BAFF levels have been reported to inversely correlate with BAFF-R expression on peripheral lymphocytes [28]. As GPA patients are known to have elevated levels of circulating BAFF [20, 21, 29], we hypothesized that the decreased BAFF-R expression is related to the increased circulating BAFF levels. BAFF was measured in plasma of patients and HC and correlated with the BAFF-R level on B cells. We found a significant negative correlation between the level of circulating BAFF and the level of BAFF-R expressed on the peripheral B cells (Spearman r=-0.6648, P<0.0001) (figure 4D).

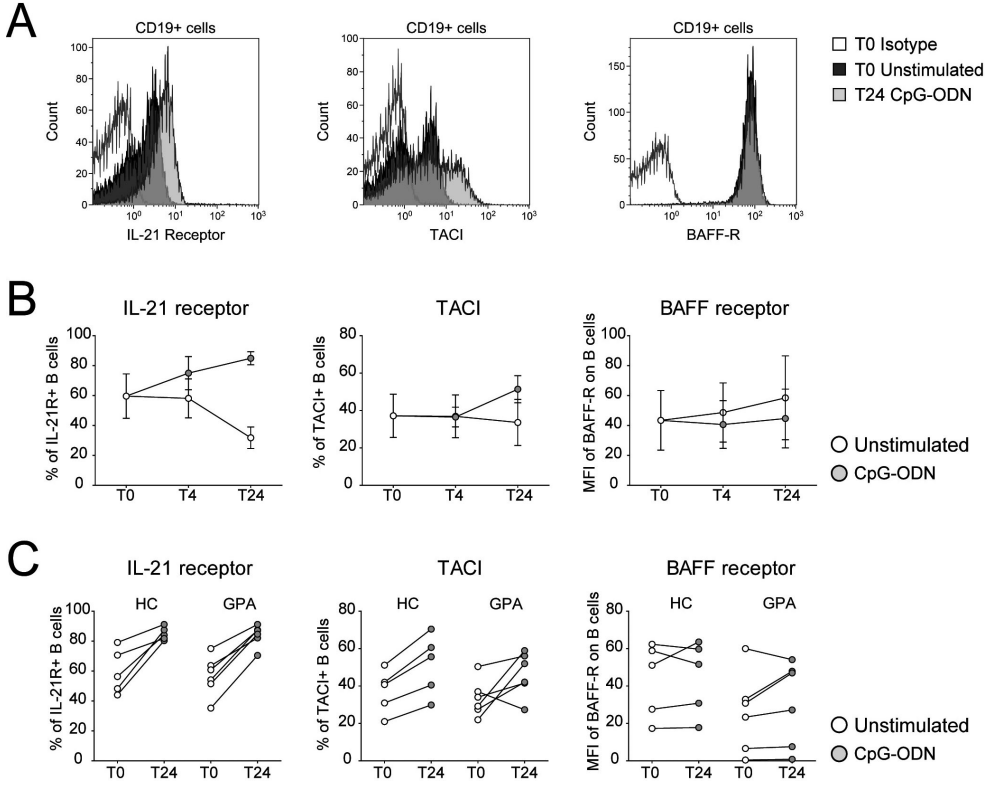


Figure 3. CpG-ODN increases IL-21R and TACI, but not BAFF-R expression on B cells. (A) Representative histograms from 1 HC, demonstrating increase in IL-21R+ and TACI+ B cells after 24 hour stimulation with CpG-ODN in comparison to the baseline expression. (B) PBMC were stimulated with CpG-ODN for 4 or 24 hours and the expression of IL-21R, TACI, and BAFF-R was measured by flow cytometry. Graphs show data from 5 HC and the data are plotted as mean±SD. (C) PBMC from 5 HC and 6 GPA patients were stimulated with CpG-ODN for 24 hours and the alterations in IL-21R, TACI, and BAFF-R expression were measured by flow cytometry.

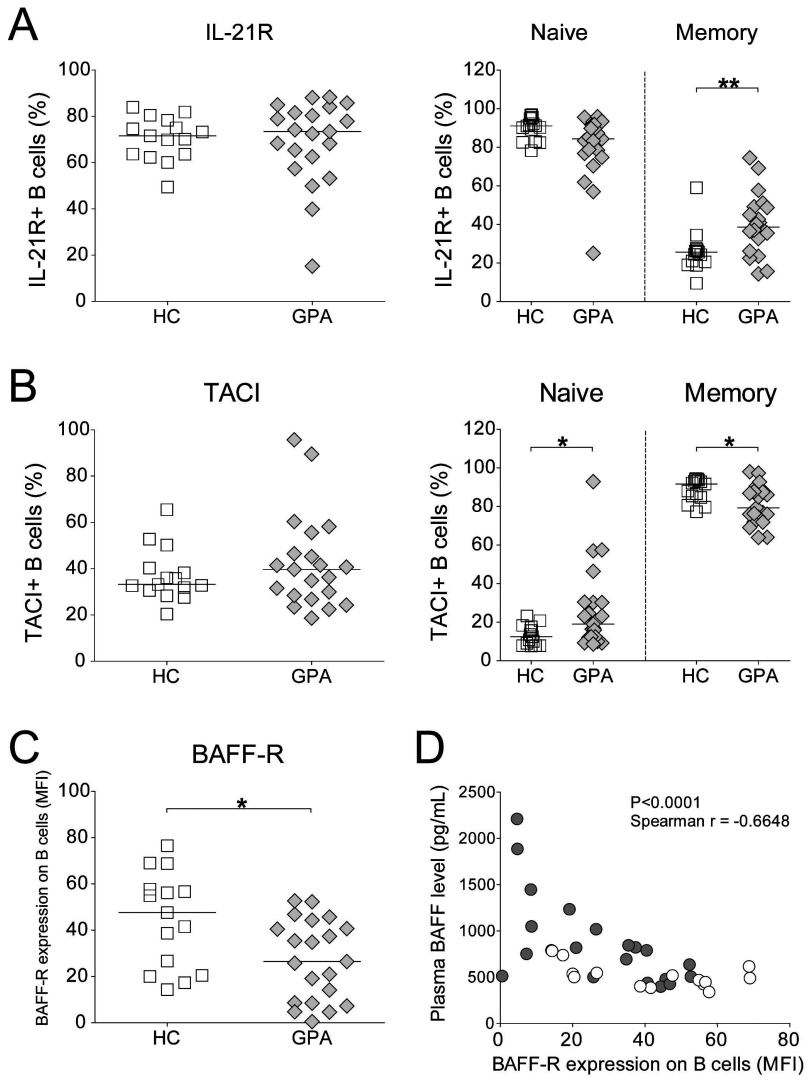


Figure 4. IL-21R, TACI and BAFF-R expression on B cells in GPA patients and HC. The percentages of (**A**) IL-21R+ and (**B**) TACI+ B cells and the receptor distribution on naïve and memory B cell subsets were assessed in HC and GPA patients. (**C**) The BAFF-R expression level on B cells is expressed as MFI. (**D**) Plasma BAFF levels were measured by ELISA; the plasma samples were collected on the same day when BAFF-R level on B cells was measured. The open circles indicate HC; the grey circles represent GPA patients. (**A-D**) The scatter plots show data from 15 HC and 21 GPA patients. In (**A-C**) graphs the horizontal lines indicate the median value. *P<0.05; **P<0.001.

DISCUSSION

In GPA pathogenesis, ANCA are considered pivotal mediators of the vasculitic damage. In the present study, we studied factors potentially involved in the

production of PR3-ANCA. Here, we show that the TLR9 agonist CpG-ODN synergizes with BAFF and IL-21 and enhances the production of PR3-ANCA, demonstrating that both endogenous and exogenous factors are effectively involved in (auto)-antibody production in GPA patients.

The combined treatment with BAFF, IL-21 and CpG-ODN efficiently induced B cell proliferation and plasma cell formation when compared to unstimulated cells. However, these effects were mainly mediated by CpG-ODN as stimulation with BAFF, IL-21, and CpG-ODN was not superior to stimulation with CpG-ODN alone, confirming previously reported effects of CpG-ODN on B cell proliferation [30-32]. A recent study by Abu-Rish *et al.* demonstrated that pre-treatment with CpG-ODN sensitizes B cells for stimulation with exogenous BAFF, resulting in markedly increased B cell proliferation when compared to cells stimulated with either CpG-ODN or BAFF alone [33]. Although we did not observe synergistic effect of BAFF addition to the cultures, in our experimental setting cells were stimulated with CpG-ODN and BAFF simultaneously, which could explain the differences in the experimental outcome.

We observed that stimulation with CpG-ODN up-regulated IL-21R on B cells, which is in concordance with a study by Ruffin *et al.* who demonstrated TLR-mediated induction of IL-21R on B cells. In our study, the observed increase in IL-21R was mainly due to increased expression on memory B cells, which, in the non-activated state, have a moderate expression level of IL-21R when compared to naive B cells. When IL-21R expression was assessed on circulating B cell subsets, GPA patients had an increased percentage of IL-21R+ memory B cells, indicating that antigen-experienced B cells present in the circulation of GPA patients have been activated and consequently might be more susceptible for stimulation through the IL-21R.

CpG-ODN also affected TACI, but not BAFF-R expression on B cells. TACI was mainly expressed on memory B cells whereas it was up-regulated on naive B cells after stimulation with CpG-ODN. These observations are in line with previous studies reporting induction of TACI expression on activated B cells [33, 34]. Remarkably, GPA patients displayed a decreased frequency of circulating TACI-expressing memory B cells. TACI is an inhibitory receptor, which under physiological conditions provides negative feedback signals to activated cells to circumvent their excessive expansion [34]. Although we have not studied the biological significance of diminished TACI expression, one might speculate that in GPA patients memory B cells receive insufficient inhibitory signals and might be more prone to uncontrolled activation.

Circulating B cells from GPA patients had lower expression of BAFF-R when compared to HC. In line with previous studies by Sellam *et al.* in SLE and SS patients, the decreased BAFF-R expression on B cells was strongly associated with increased BAFF levels in circulation. Interestingly, the authors also reported that the decreased BAFF-R expression on peripheral lymphocytes was associated with increased disease activity in SLE and SS patients [28]. One of the GPA patients included in our study had almost no detectable expression of BAFF-R on the circulating B lymphocytes when cells were stained using whole blood or directly after PBMC isolation. However, after 24 hour

culture *in vitro* without additional stimulation, B cells re-expressed BAFF-R, suggesting that BAFF-R has been internalized due to high BAFF levels present in the circulation.

Elevated BAFF levels, increased frequency of IL-21-producing T cells or circulating IL-21 have been reported in various autoimmune conditions, including GPA [14, 35, 36]. Over-expression of BAFF or IL-21 in animal models leads to the development of a lupus-like condition whereas blockade of these cytokines ameliorates the disease severity [17, 37]. Based on these observations, BAFF, IL-21 and/or their receptors could constitute attractive therapeutic targets to inhibit pathogenic B cell functions in autoimmune disorders. A monoclonal antibody inhibiting BAFF has been approved for the treatment of SLE patients [38], and ongoing clinical trials also aim to test the efficacy of anti-BAFF therapy in vasculitis patients [39]. This might be of particular interest, as the level of circulating BAFF is known to increase after B cell depletion therapy [40], which is also being used in GPA patients [41]. As the B cells reconstitute in a BAFF-enriched environment, therapy that regulates BAFF levels in circulation might be useful to prevent aberrant activation of newly formed B cells. More recently, a monoclonal antibody that interferes with IL-21 signaling has been developed and is being tested in a clinical trial as a possible treatment for rheumatoid arthritis (RA) [42]. The data of the current study provide additional evidence that blockade of BAFF and/or IL-21 might be a beneficial strategy for amelioration of autoantibody-mediated diseases.

Our study primarily focused on patients in clinical remission and only a limited number of patients with active disease (n=6) were included to establish ANCA production *in vitro*. As ANCA have a major role in the effector phase of GPA [2], the factors that contribute to the production of autoantibodies potentially could exacerbate disease activity or promote the occurrence of relapses.

In conclusion, we have demonstrated that endogenous B cell stimuli and bacterial products can contribute to autoreactive B cell activation in GPA patients in remission, emphasizing the contribution of infectious agents in re-activation of autoantibody production. Therapeutic strategies which regulate B cell activation might be useful to sustain stable remission in GPA patients.

ACKNOWLEDGEMENTS

We thank Dr. Caroline Roozendaal and Jetske Anema from the Laboratory Center (University Medical Center Groningen) for their assistance with quantification of PR3-ANCA. Research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 261382 and the Dutch Arthritis Foundation (Reumafonds project number 11-1-405).

REFERENCES

1. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, Flores-Suarez LF, Gross WL, Guillevin L, Hagen EC, Hoffman GS, Jayne DR, Kallenberg CG, Lamprecht P, Langford CA, Luqmani RA, Mahr AD, Matteson EL, Merkel PA, Ozen S, Pusey CD, Rasmussen N, Rees AJ, Scott DG, Specks U, Stone JH, Takahashi K, Watts RA: **2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides.** *Arthritis Rheum* 2013, **65**(1):1-11.
2. Jennette JC, Falk RJ, Hu P, Xiao H: **Pathogenesis of antineutrophil cytoplasmic autoantibody-associated small-vessel vasculitis.** *Annu Rev Pathol* 2013, **8**:139-160.
3. Falk RJ, Terrell RS, Charles LA, Jennette JC: **Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro.** *Proc Natl Acad Sci U S A* 1990, **87**(11):4115-4119.
4. Mulder AH, Heeringa P, Brouwer E, Limburg PC, Kallenberg CG: **Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA): a Fc gamma RII-dependent process.** *Clin Exp Immunol* 1994, **98**(2):270-278.
5. Kettritz R, Jennette JC, Falk RJ: **Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils.** *J Am Soc Nephrol* 1997, **8**(3):386-394.
6. Little MA, Al-Ani B, Ren S, Al-Nuaimi H, Leite M,Jr, Alpers CE, Savage CO, Duffield JS: **Anti-proteinase 3 anti-neutrophil cytoplasm autoantibodies recapitulate systemic vasculitis in mice with a humanized immune system.** *PLoS One* 2012, **7**(1):e28626.
7. Stegeman CA, Tervaert JW, Sluiter WJ, Manson WL, de Jong PE, Kallenberg CG: **Association of chronic nasal carriage of Staphylococcus aureus and higher relapse rates in Wegener granulomatosis.** *Ann Intern Med* 1994, **120**(1):12-17.
8. Stegeman CA, Tervaert JW, de Jong PE, Kallenberg CG: **Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis. Dutch Co-Trimoxazole Wegener Study Group.** *N Engl J Med* 1996, **335**(1):16-20.
9. Hurtado PR, Jeffs L, Nitschke J, Patel M, Sarvestani G, Cassidy J, Hissaria P, Gillis D, Peh CA: **CpG oligodeoxynucleotide stimulates production of anti-neutrophil cytoplasmic antibodies in ANCA associated vasculitis.** *BMC Immunol* 2008, **9**(1471-2172):34.
10. Tadema H, Abdulahad WH, Lepse N, Stegeman CA, Kallenberg CG, Heeringa P: **Bacterial DNA motifs trigger ANCA production in ANCA-associated vasculitis in remission.** *Rheumatology (Oxford)* 2011, **50**(4):689-696.
11. Brouwer E, Stegeman CA, Huitema MG, Limburg PC, Kallenberg CG: **T cell reactivity to proteinase 3 and myeloperoxidase in patients with Wegener's granulomatosis (WG).** *Clin Exp Immunol* 1994, **98**(3):448-453.
12. Brouwer E, Tervaert JW, Horst G, Huitema MG, van der Giessen M, Limburg PC, Kallenberg CG: **Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis and clinically related disorders.** *Clin Exp Immunol* 1991, **83**(3):379-386.
13. Tangye SG, Ma CS, Brink R, Deenick EK: **The good, the bad and the ugly - TFH cells in human health and disease.** *Nat Rev Immunol* 2013, **13**(6):412-426.
14. Abdulahad WH, Lepse N, Stegeman CA, Huitema MG, Doornbos-van der Meer B, Tadema HH, Rutgers A, Limburg PC, Kallenberg CG, Heeringa P: **Increased frequency of circulating IL-21 producing Th-cells in patients with granulomatosis with polyangiitis.** *Arthritis Res Ther* 2013, **15**(3):R70.
15. Karnell JL, Ettinger R: **The Interplay of IL-21 and BAFF in the Formation and Maintenance of Human B Cell Memory.** *Front Immunol* 2012, **3**:2.
16. Ettinger R, Sims GP, Robbins R, Withers D, Fischer RT, Grammer AC, Kuchen S, Lipsky PE: **IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells.** *J Immunol* 2007, **178**(5):2872-2882.
17. Groom JR, Fletcher CA, Walters SN, Grey ST, Watt SV, Sweet MJ, Smyth MJ, Mackay CR, Mackay F: **BAFF and MyD88 signals promote a lupuslike disease independent of T cells.** *J Exp Med* 2007, **204**(0022-1007; 0022-1007; 8):1959-1971.

18. Zhang J, Roschke V, Baker KP, Wang Z, Alarcon GS, Fessler BJ, Bastian H, Kimberly RP, Zhou T: **Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus.** *J Immunol* 2001, **166**(1):6-10.
19. Mariette X, Roux S, Zhang J, Bengoufa D, Lavie F, Zhou T, Kimberly R: **The level of BLyS (BAFF) correlates with the titre of autoantibodies in human Sjogren's syndrome.** *Ann Rheum Dis* 2003, **62**(2):168-171.
20. Sanders JS, Huitma MG, Kallenberg CG, Stegeman CA: **Plasma levels of soluble interleukin 2 receptor, soluble CD30, interleukin 10 and B cell activator of the tumour necrosis factor family during follow-up in vasculitis associated with proteinase 3-antineutrophil cytoplasmic antibodies: associations with disease activity and relapse.** *Ann Rheum Dis* 2006, **65**(11):1484-1489.
21. Bader L, Koldingsnes W, Nossent J: **B-lymphocyte activating factor levels are increased in patients with Wegener's granulomatosis and inversely correlated with ANCA titer.** *Clin Rheumatol* 2010, **29**(9):1031-1035.
22. Jennette JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, Hagen EC, Hoffman GS, Hunder GG, Kallenberg CG: **Nomenclature of systemic vasculitides. Proposal of an international consensus conference.** *Arthritis Rheum* 1994, **37**(2):187-192.
23. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, Calabrese LH, Fries JF, Lie JT, Lightfoot RW,Jr: **The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis.** *Arthritis Rheum* 1990, **33**(8):1101-1107.
24. Geffroy-Luseau A, Chiron D, Descamps G, Jego G, Amiot M, Pellat-Deceunynck C: **TLR9 ligand induces the generation of CD20+ plasmablasts and plasma cells from CD27+ memory B-cells.** *Front Immunol* 2011, **2**:83.
25. Katsenelson N, Kanswal S, Puig M, Mostowski H, Verthelyi D, Akkoyunlu M: **Synthetic CpG oligodeoxynucleotides augment BAFF- and APRIL-mediated immunoglobulin secretion.** *Eur J Immunol* 2007, **37**(7):1785-1795.
26. Buchanan RM, Popowych Y, Arsic N, Townsend HG, Mutwiri GK, Potter AA, Babiuk LA, Griebel PJ, Wilson HL: **B-cell activating factor (BAFF) promotes CpG ODN-induced B cell activation and proliferation.** *Cell Immunol* 2011, **271**(1):16-28.
27. Darce JR, Arendt BK, Wu X, Jelinek DF: **Regulated expression of BAFF-binding receptors during human B cell differentiation.** *J Immunol* 2007, **179**(11):7276-7286.
28. Sellam J, Miceli-Richard C, Gottenberg JE, Ittah M, Lavie F, Lacabaratz C, Gestermann N, Proust A, Lambotte O, Mariette X: **Decreased B cell activating factor receptor expression on peripheral lymphocytes associated with increased disease activity in primary Sjogren's syndrome and systemic lupus erythematosus.** *Ann Rheum Dis* 2007, **66**(6):790-797.
29. Nagai M, Hirayama K, Ebihara I, Shimohata H, Kobayashi M, Koyama A: **Serum Levels of BAFF and APRIL in Myeloperoxidase Anti-Neutrophil Cytoplasmic Autoantibody-Associated Renal Vasculitis: Association with Disease Activity.** *Nephron Clin Pract* 2011, **118**(4):c339-c345.
30. Hartmann G, Krieg AM: **Mechanism and function of a newly identified CpG DNA motif in human primary B cells.** *J Immunol* 2000, **164**(2):944-953.
31. Huggins J, Pellegrin T, Felgar RE, Wei C, Brown M, Zheng B, Milner EC, Bernstein SH, Sanz I, Zand MS: **CpG DNA activation and plasma-cell differentiation of CD27- naive human B cells.** *Blood* 2007, **109**(4):1611-1619.
32. Jiang W, Lederman MM, Harding CV, Rodriguez B, Mohner RJ, Sieg SF: **TLR9 stimulation drives naive B cells to proliferate and to attain enhanced antigen presenting function.** *Eur J Immunol* 2007, **37**(8):2205-2213.
33. Abu-Rish EY, Amrani Y, Browning MJ: **Toll-like receptor 9 activation induces expression of membrane-bound B-cell activating factor (BAFF) on human B cells and leads to increased proliferation in response to both soluble and membrane-bound BAFF.** *Rheumatology (Oxford)* 2013, **52**(7):1190-1201.
34. Mackay F, Schneider P: **TACI, an enigmatic BAFF/APRIL receptor, with new unappreciated biochemical and biological properties.** *Cytokine Growth Factor Rev* 2008, **19**(1359-6101; 3-4):263-276.
35. Dolff S, Abdulahad WH, Westra J, Doornbos-van der Meer B, Limburg PC, Kallenberg CG, Bijl M: **Increase in IL-21 producing T-cells in patients with systemic lupus erythematosus.** *Arthritis Res Ther* 2011, **13**(5):R157.

36. Ma J, Zhu C, Ma B, Tian J, Baidoo SE, Mao C, Wu W, Chen J, Tong J, Yang M, Jiao Z, Xu H, Lu L, Wang S: **Increased frequency of circulating follicular helper T cells in patients with rheumatoid arthritis.** *Clin Dev Immunol* 2012, **2012**:827480.
37. Ozaki K, Spolski R, Ettinger R, Kim HP, Wang G, Qi CF, Hwu P, Shaffer DJ, Akilesh S, Roopenian DC, Morse HC,3rd, Lipsky PE, Leonard WJ: **Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6.** *J Immunol* 2004, **173**(9):5361-5371.
38. Lamore R,3rd, Parmar S, Patel K, Hilas O: **Belimumab (benlysta): a breakthrough therapy for systemic lupus erythematosus.** *P T* 2012, **37**(4):212-226.
39. [http://clinicaltrials.gov/show/NCT01663623]
40. Pollard RP, Abdulahad WH, Vissink A, Hamza N, Burgerhof JG, Meijer JM, Visser A, Huitema MG, Spijkervet FK, Kallenberg CG, Bootsma H, Kroese FG: **Serum levels of BAFF, but not APRIL, are increased after rituximab treatment in patients with primary Sjogren's syndrome: data from a placebo-controlled clinical trial.** *Ann Rheum Dis* 2013, **72**(1):146-148.
41. Specks U, Merkel PA, Seo P, Spiera R, Langford CA, Hoffman GS, Kallenberg CG, St Clair EW, Fessler BJ, Ding L, Viviano L, Tchao NK, Phippard DJ, Asare AL, Lim N, Ikle D, Jepson B, Brunetta P, Allen NB, Fervenza FC, Geetha D, Keogh K, Kissin EY, Monach PA, Peikert T, Stegeman C, Ytterberg SR, Mueller M, Sejismundo LP, Mieras K, Stone JH, RAVE-ITN Research Group: **Efficacy of remission-induction regimens for ANCA-associated vasculitis.** *N Engl J Med* 2013, **369**(5):417-427.
42. [htp://clinicaltrials.gov/show/NCT01208506]

CHAPTER 5

ALTERED B CELL BALANCE, BUT UNAFFECTED B CELL CAPACITY TO LIMIT MONOCYTE ACTIVATION IN ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODY-ASSOCIATED VASCULITIS IN REMISSION

Nikola Lepse, Wayel H. Abdulahad, Abraham Rutgers, Cees G. M. Kallenberg, Coen A. Stegeman and Peter Heeringa

PUBLISHED
RHEUMATOLOGY (OXFORD) DOI:10.1093/RHEUMATOLOGY/KEU149

ABSTRACT

Objective

Regulatory B cells (B_{REGS}) constitute a subset of B cells with immunomodulatory properties. Numerical and functional alterations in the B_{REG} compartment have been associated with autoimmunity. The aim of this study was to assess the frequency and function of B_{REGS} in patients with ANCA-associated vasculitis (AAV).

Methods

B cell subsets were determined in the peripheral blood of 48 AAV patients (12 active, 36 in remission) and 41 healthy controls (HCs) by flow cytometry. B_{REGS} were defined within the $CD19^+$ population as $CD24^{hi}CD38^{hi}$ or $CD24^{hi}CD27^+$ cells. The percentage of IL-10-positive B cells in circulation was analysed by flow cytometry. Sorted CD19+ B cells were co-cultured with monocytes to evaluate their capacity to inhibit monocyte TNF-α production upon lipopolysaccharide stimulation.

Results

The frequency of circulating $CD19^+CD24^{hi}CD38^{hi}$ cells was not different in AAV patients in remission compared with HCs, but was decreased in patients with active disease (mean in HCs 5.5% (S.D. 1.6) vs active 3.8% (S.D. 2.8), P = 0.0104). Furthermore, the percentage of $CD19^+CD24^{hi}CD27^+$ cells was significantly decreased in both remission and active patients when compared with HCs (HCs 15.0% (S.D. 9.3) vs remission 6.6% (S.D. 4.4) (P<0.0001) vs active 6.4% (S.D. 6.2) (P = 0.0006)). The frequency of IL-10-positive B cells was comparable between patients and HCs. B cells from AAV patients suppressed monocyte TNF-α production to a similar extent to cells from HCs.

Conclusions

Based on immunophenotypic classification, B_{REGS} are numerically diminished in AAV patients. However, B cell function in terms of IL-10 production and their capacity to suppress monocyte activation is not compromised in AAV patients in remission.

INTRODUCTION

ANCA-associated vasculitides (AAV) comprise a group of severe inflammatory diseases characterized by the presence of autoantibodies that target specific neutrophil cytoplasmic proteins, in particular PR3 and MPO [1]. AAV primarily affect small- to medium-sized blood vessels and based on clinical and pathological features, three disease entities can be distinguished - granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic GPA [2, 3].

Recently, two major clinical trials demonstrated that treatment with the B cell-depleting antibody rituximab is efficacious in inducing disease remission in AAV [4, 5], and continuous B cell depletion might be a useful strategy for remission maintenance [6, 7]. These results underscore the crucial role of B cells in AAV pathogenesis. However, the underlying mechanisms are complex and the precise role of B cells in AAV is incompletely understood.

Under physiological circumstances, B cells are critical players in the regulation of immune responses, providing protection against infection without causing overt damage to the host. This is accomplished through a finely regulated balance between B cell effector and regulatory functions exerted in an antibody-dependent, as well as an antibody-independent fashion [8]. An increasing body of evidence indicates that in autoimmunity the interplay between pathogenic and protective B cell functions is dysregulated. In this context, the identification of regulatory B cells (B_{REGS}) in both mice and humans has gained considerable interest in the autoimmunity field. B_{REGS} are defined by their capacity to suppress immune responses primarily via provision of IL-10 [9]. A number of studies in mouse models of autoimmunity have shown that IL-10-producing B cells were capable of suppressing disease development [10-13]. IL-10-producing B_{REGS} and their possible contribution to autoimmune responses have also been studied in humans. Identification of circulating B_{REGS} in humans has been suggested based on high expression of CD24 in combination with expression of either CD38 [14] or CD27 [15] ($CD19+CD24^{high}CD38^{high}$ or $CD19+CD24^{high}CD27+$). Interestingly, dysregulated frequencies and/or defective function of B_{REGS} have been documented in autoimmune diseases, such as multiple sclerosis (MS) [16, 17], SLE [14] and RA [18]. $CD19^{+}CD24^{high}CD38^{high}$ cells from normal individuals were shown to suppress IFN-γ production by CD4+ T cells, while in SLE patients B cell suppressive function was reduced [14]. Recent data also indicate that $CD19^{+}CD24^{high}CD38^{high}$ cells inhibit the differentiation of naive T cells into Th1 or Th17 cells and promote the development of regulatory T cells [18]. These effects were mediated in part by provision of IL-10 and were found to be partially impaired in RA patients. Others have reported that IL-10-producing B_{REGS} reside in the $CD19+CD24^{hi}CD27+$ subset, which can suppress TNF-α production by monocytes [15].

Overall, current evidence indicates that B_{REGS} play an important immune regulatory role and suggests that alterations in either number or function of B_{REGS} contribute to pathogenic immune responses in autoimmune diseases. However, so far data on B_{REG} frequency and/or function in AAV patients is limited. In the present study, the frequencies of B_{REGS} in

peripheral blood were assessed in a cohort of AAV patients and healthy individuals based on cell surface markers. In addition, the capacity of peripheral B cells to produce IL-10 and their ability to suppress monocyte TNF-α production were determined.

PATIENTS AND METHODS

Study population

Peripheral blood was collected from 48 PR3-AAV patients (mean age 57.8±13.6 years) and 41 age- and sex-matched healthy controls (HCs; mean age 54.5±7.4 years). The characteristics of patients and HCs included in the study are described in table 1. Thirty-two out of 48 patients (67%) received oral cyclophosphamide (2 mg/kg/day) and prednisolone (60 mg/day) for induction therapy. None of the patients had received rituximab prior to inclusion in this study. Patients were at least 3 months without cyclophosphamide treatment before inclusion in this study. The diagnosis of GPA or MPA was based on the definitions outlined in the Chapel Hill Consensus Conference [19], and GPA patients fulfilled the classification criteria of the American College of Rheumatology (ACR) [19, 20]. Samples were obtained in compliance with the Declaration of Helsinki. All subjects provided informed consent, and the study was approved by the Medical Ethics Committee of the University Medical Center Groningen, University of Groningen (NL).

Flow cytometry for B cell phenotype analysis

The expression of CD24, CD27 and CD38 by peripheral blood B lymphocytes was determined by flow cytometry. Briefly, freshly drawn EDTA blood was washed twice with PBS supplemented with 1% BSA to remove the plasma. After washing, cells were suspended in PBS plus 1% BSA to the original volume, and 100 µl of the cell suspension was incubated with anti-human CD19-eFluor-450 (eBioscience, San Diego, CA, USA; clone HIB19), anti-human CD24-FITC (BD Biosciences, San Jose, CA, USA; clone ML5), anti-human CD27-APC-eFluor-780 (eBioscience, clone O323), anti-human CD38-PE-Cy7 (eBioscience, clone HIT2), or the corresponding isotype control antibodies for 15 minutes in the dark. The red blood cells were lysed with FACS Lysing Solution (BD Biosciences). Cells were washed with PBS plus 1% BSA and analyzed with a BD™ LSR-II flow cytometer (BD, Franklin Lakes, NJ, USA). The data were analysed using Kaluza 1.2 Flow Analysis Software (Beckman Coulter, Brea, CA, USA).

Detection of IL-10-producing B cells by flow cytometry

According to the previously described methodology [15], IL-10-producing B cells were quantified using two approaches. Based on the method used, the IL-10-positive B cells are termed either B10 or B10pro cells. For detection of B10 cells, heparinized blood was diluted 1:1 with RPMI 1640 (Lonza, Basel, Switzerland), and stimulated for 4h at 37 °C with 50 ng/mL of phorbol myristate acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) and 2 mM calcium ionophore (Sigma-Aldrich) in the presence of 10 µg/mL brefeldin A (BFA; Sigma-Aldrich). At the end of the culture, the red blood cells were lysed with ammonium

Table 1. Characteristics of the study population

Characteristics	HCs	AAV Remission	AAV Active
Number of subjects, *n* (males, %)	41 (59%)	36 (61%)	12 (58%)
Age, mean (range), years	54.5 (40-71)	57.8 (27-81)	57.9 (31-78)
Diagnosis			
GPA		33	12
MPA		3	0
Disease form			
Localized		3	3
Generalized		33	9
Frequency of clinical manifestations, n (%)			
General		15 (42%)	6 (50%)
Cutaneous		8 (22%)	2 (17%)
Mucosal membranes / eyes		6 (17%)	4 (33%)
ENT		25 (69%)	8 (67%)
Chest		15 (42%)	6 (50%)
Cardiovascular		2 (6%)	2 (17%)
Abdominal		3 (8%)	0 (0%)
Renal		22 (61%)	7 (58%)
Nervous system		10 (28%)	3 (25%)
PR3-ANCA titer, median (range)		80 (20-640)	160 (0-640)
Induction therapy			
CYC (2 mg/kg/day), Pred (60 mg//day)		23	9
CYC, Pred, MP		4	0
CYC, Pred, plasma exchange		3	1
Co-trimoxazole		3	1
CYC, Pred, MP, plasma exchange		1	0
AZA, Pred		1	0
MTX, Pred		0	1
Pred		1	0
Treatment at the time of sampling			
AZA (25 - 100 mg/day)		4	1
Pred (5 – 10 mg/day)		5	2
AZA (100 mg/day) / Pred (5 mg/day)		1	0
Pred (10 mg/day) /MMF (2000 mg/day)		0	1
No immunosuppressive therapy		26	8
BVAS, median (range)		0	14.5 (2 – 20)

AAV, ANCA-associated vasculitis; ANCA, anti-neutrophil cytoplasmic autoantibodies; AZA, azathioprine; BVAS, Birmingham Vasculitis Activity Score; CYC, cyclophosphamide; GPA, granulomatosis with polyangiitis; HC, healthy controls; MMF, mycophenolate mofetil; MP, methylprednisolone; MPA, microscopic polyangiitis; MTX, methotrexate; PR3, proteinase 3; pred, prednisolone.

chloride buffer, and the remaining cells were washed with PBS plus 5% fetal calf serum (FCS; Lonza). Then cells were stained with anti-human CD19-eFluor-450 and anti-human CD22-PE-Cy5 (BD Biosciences, clone HIB22) for 15 minutes in the dark. The cells were then fixed and permeabilized using the Fix&Perm kit (Invitrogen, Life Technologies, Grand Island, NY, USA), and incubated with anti-human IL-10-PE (BioLegend, San Diego, CA, USA; clone JES3-9D7) to stain for intracellular IL-10 expression.

For induction of B10pro cells, peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640 (with 50 µg/ml gentamicin (GIBCO, Life Technologies) and 10% FCS) at a concentration of $1x10^6$/mL. Cells were left untreated or were stimulated with CpG-ODN-2006 (500 ng/mL; Hycult Biotech, Uden, the Netherlands) for 72h. In the last 5h the cells were re-stimulated with PMA (50 ng/mL) and calcium ionophore (2 mM) in the presence of BFA (10 µg/mL). Cells were washed with PBS plus 5% FCS and stained using the same protocol as described above for B10 cells. All samples were analysed with an LSR-II flow cytometer, and the data were processed using Kaluza 1.2 Flow Analysis Software.

Monocyte suppression assay

B cell suppressive capacity was evaluated as described before [15]. PBMCs were isolated from heparinized blood, and cells were labelled with anti-human CD19-eFluor-450. CD19+ cells were sorted with fluorescence activated cell sorter (FACS; MoFlo™, Beckman Coulter); based on the post-sort purity analysis, the obtained population was > 95% pure. Sorted B cells were cultured in the presence or absence of CpG-ODN 2006 (10 µg/mL) and anti-CD40 (1 µg/mL; eBioscience, clone 5C3) for 24h. Monocytes were isolated using the BD IMag™ Monocyte Enrichment Set – DM (BD Biosciences), according to the manufacturer's instructions. After 24h, B cells were washed and monocytes were added in a ratio of 1:1. B cells and monocytes were co-cultured for 24h. In the last 4h, 1 µg/mL of lipopolysaccharide (LPS; Ultra pure; InvivoGen, San Diego, CA, USA) and BFA (10 µg/mL) were added to induce TNF-α production in monocytes. At the end of the experiment, cells were washed with PBS plus 5% FCS, and stained with anti-human CD14-PerCP-Cy5.5 (BD Biosciences, clone M5E2) for 15 minutes in the dark. Then cells were fixed, permeabilized (Fix&Perm), and stained with anti-human TNF-α APC (BD Biosciences, clone MAb11). Samples were analysed with FACSCalibur (BD Biosciences), and Kaluza 1.2 Flow Analysis Software was used for data analysis.

Statistical analysis

Data represent median values unless stated otherwise. For comparison of more than two groups, one-way analysis of variance (ANOVA) was used if the data were normally distributed, and Kruskal-Wallis test was applied if the data had a non-Gaussian distribution, as determined by the D'Agostino and Pearson omnibus normality test. If a significant difference was found, further testing was done using an unpaired t test or Mann-Whitney test for data with Gaussian and non-Gaussian distribution, respectively. A P value < 0.05 was considered statistically significant. *P<0.05; **P<0.001; ***P<0.0001.

RESULTS

Altered distribution of B cell subsets in AAV patients

Phenotypic characterization of the circulating B cell population was performed in 48 AAV patients and 41 age- and gender-matched HCs. Lymphocytes were gated from the forward-side scatter based on their size and expression of CD19 was used to separate B cells (figure 1A). Transitional, naive and memory B cells were characterized based on expression of CD27 and CD38 (figure 1B). Surface expression of CD24 in combination with CD27 or CD38 was used to distinguish two proposed B_{REG} subsets (figures 1C and 1D).

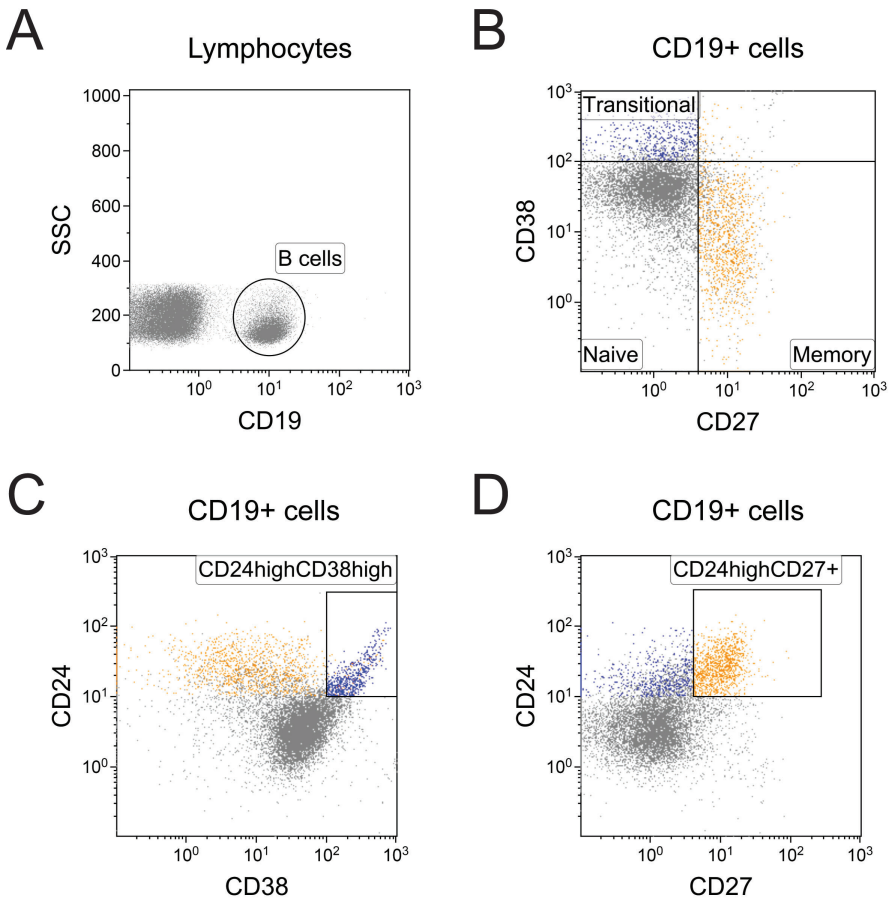


Figure 1. Gating strategies for B cell subsets. Representative flow cytometry dot plots from 1 healthy individual. Lymphocytes were gated based on the forward-side scatter profile. (**A**) Within the lymphocyte population, B cells were distinguished by expression of CD19. (**B**) Cell surface markers CD27 and CD38 were used to further characterize B cell subpopulations. Transitional B cells were CD19+CD27-CD38^{high}, naive B cells were defined as CD19+CD27-CD38$^{-/low}$, and memory B cells were CD19+CD27+CD38$^{-/low}$. The two putative regulatory B cell subsets were defined as CD19+CD24^{high}CD38^{high} (**C**) or CD19+CD24^{high}CD27+ (**D**).

The percentage of CD19+ B cells within the circulating lymphocyte population did not differ between AAV patients and HCs (figure 2A). AAV patients with active disease had a decreased percentage of transitional B cells when compared with HCs or patients in remission (figure 2B). Additionally, AAV patients had an increased percentage of naive B cells independent of disease activity (figure 2C). In line with previous studies [21], we found a significantly decreased proportion of circulating memory B cells in AAV patients when compared with HCs (figure 2D).

Further, we characterized the $CD19^{+}CD24^{high}CD38^{high}$ and $CD19^{+}CD24^{high}CD27^{+}$ subsets which have been reported to have immunoregulatory properties in humans [14, 15]. Interestingly, the $CD19^{+}CD24^{high}CD38^{high}$ population was overlapping with the transitional B cell subset, whereas $CD19^{+}CD24^{high}CD27^{+}$ cells represented a subset of the memory B cell population (figures 1B-1D). Thus the proposed regulatory subsets represent two distinct populations. The proportion of $CD19^{+}CD24^{high}CD38^{high}$ cells was not different between HCs and AAV patients in remission, but was significantly decreased in patients with active disease when compared with HCs and patients in remission (figure 2E). Also, the percentage of $CD19^{+}CD24^{high}CD27^{+}$ cells was diminished in both remission and active patients when compared with HCs (figure 2F).

Effect of treatment on B cell subset distribution

In order to rule out the effect of treatment on B cell subset distribution, we compared the percentage of total B cells and B cell subsets in untreated (n = 34) and treated (n = 14) patient groups. When patients were subdivided based on treatment but independent of disease activity, no significant differences were found in the percentage of circulating total B cells or any of the given B cell subsets. When only patients in clinical remission (n = 36) were subdivided based on treatment, treated patients (n = 10) had a significantly decreased percentage of circulating B cells, and none of the B cells subsets were significantly affected by the treatment (table 2). Additionally, we analysed the possible relationship between clinical phenotypes and the proposed B_{REG} subsets. The percentage of circulating $CD19+CD24^{high}CD38^{high}$ or $CD19+CD24^{high}CD27+$ cells was not associated with renal, ENT, chest involvement or the frequency of relapses.

The percentage of IL-10-producing B cells is not diminished in AAV patients

Previous studies have suggested the existence of a rare B cell subset (termed B10 cells) that represents ~1% of circulating B cells and is capable of producing IL-10 upon short stimulation with PMA and calcium ionophore. Additionally, a subset of peripheral blood B cells can be induced *in vitro* to become IL-10-producing B cells (B10pro cells) after prolonged stimulation with Toll-like receptor (TLR) agonists [15]. As IL-10 is considered the signature effector cytokine of B_{REGS}, we evaluated the frequency of both naturally occurring IL-10-competent B cells (B10 cells) and inducible IL-10-producing B cells (B10pro cells) in order to indirectly examine the functionality of B_{REGS} in AAV patients.

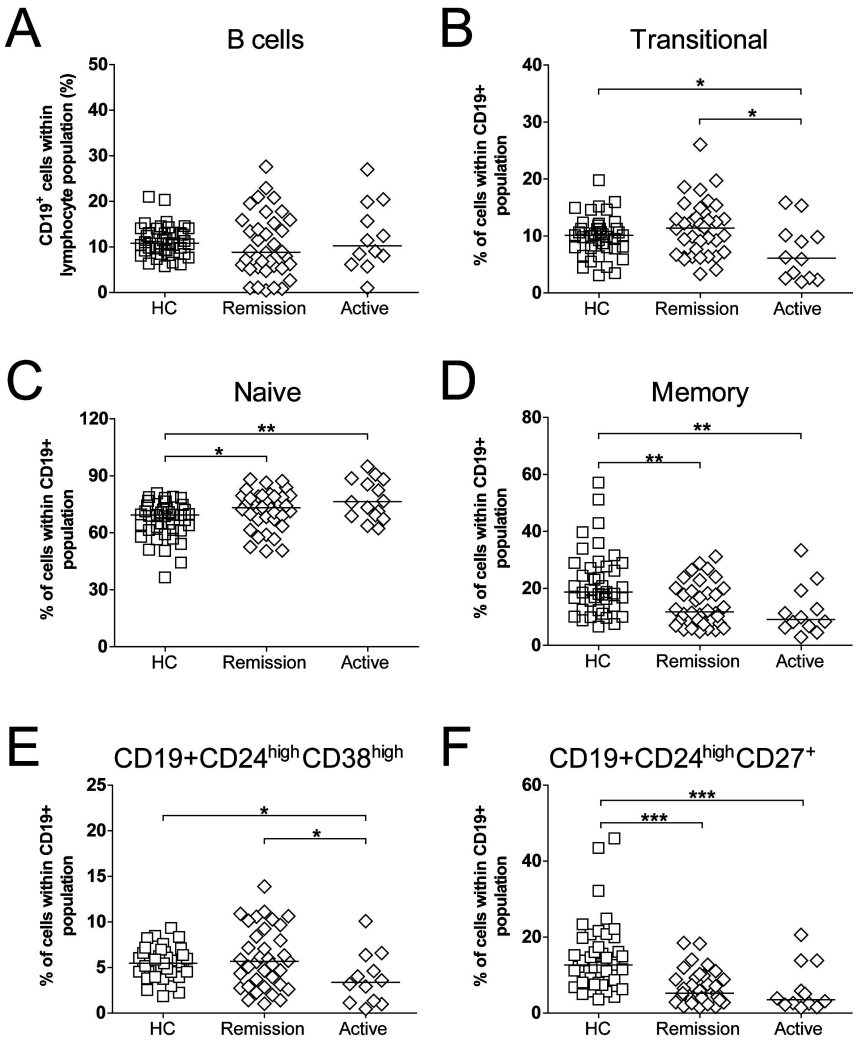


Figure 2. Characterization of circulating B cell subsets. Distribution of B cell subsets in AAV patients and HC. (A-F) Graphs represent data of 41 HC, 36 patients in clinical remission and 12 patients with active disease. Horizontal lines indicate median values. P value < 0.05 was considered statistically significant. *P<0.05; **P<0.001; ***P<0.0001.

For quantification of the B10 cells, whole blood was stimulated for 4 h with PMA and calcium ionophore (figure 3A). Stimulation in this manner induced IL-10 expression in 0.82% (range 0.41-2.03) of B cells in samples from HCs (n = 10). A similar level of B10 cells was found in AAV patients (n = 10), who had 0.82% (range 0.19-1.96) of IL-10-positive B cells (figure 3B).

To determine the frequency of the B10pro cells, PBMCs were stimulated with TLR9 ligand CpG-ODN for 3 days (figure 3C). The frequency of the inducible IL-10+

Table 2. Effect of treatment and disease activity on B cell subset distribution

	Untreated (n=34)	Treated (n=14)		Remission Untreated (n=26)	Remission Treated (n=10)	
Compartment	Median % (range)		P value	Median % (range)		P value
B cells CD19+ cells	10.2 (0.46-27.6)	6.5 (0.82-27.0)	0.0582	12.1 (0.46-27.6)	4.4 (0.82-15.9)	0.0085
Transitional B cells CD19+CD27^{-}CD38^{hi}	10.1 (1.95-26.1)	6.8 (2.6-19.7)	0.3522	12.1 (3.3-26.1)	8.4 (4.1-19.7)	0.2893
Naive B cells CD19+CD27^{-}CD38$^{-/low}$	73.8 (52.5-94.9)	72.5 (50.3-90.7)	0.5089	73.8 (52.5-88.1)	68.5 (50.3-82.8)	0.0810
Memory B cells CD19+CD27^{+}CD38$^{-/low}$	10.6 (2.9-33.4)	12.5 (4.6-31.2)	0.3825	10.6 (4.8-28.8)	18.1 (7.4-31.2)	0.0721
CD19+CD24^{hi}CD38^{hi}	5.7 (0.45-13.9)	3.6 (1.0-10.9)	0.2646	5.9 (1.2-13.9)	3.9 (1.0-10.9)	0.3469
CD19+CD24^{hi}CD27+	4.8 (1.5-20.6)	4.8 (1.4-14.1)	0.9729	4.9 (1.5-18.5)	5.8 (4.0-14.0)	0.2975

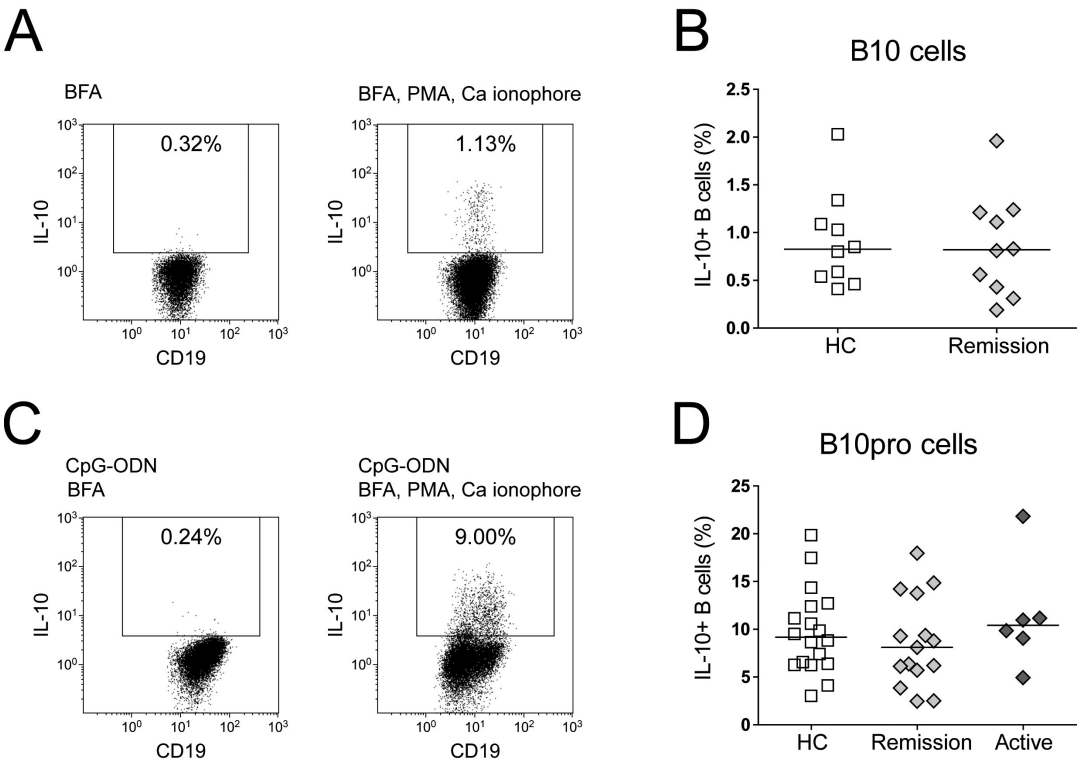


Figure 3. The percentage of IL-10-producing B cells in AAV patients and HCs. (**A and B**) For detection of B10 cells, whole blood samples were stimulated with PMA and calcium ionophore in the presence of BFA for 4h. (**C and D**) For induction of B10pro cells, PBMCs were stimulated with CpG-ODN for 3 days; PMA, calcium ionophore, and BFA were added in the final 5h of the culture. (**A and C**) Representative flow cytometry dot plots from 1 healthy individual, demonstrating the gating strategy for B10 cells (**A**) and B10pro cells (**C**). Samples incubated with BFA only were used to set the gate. (**B**) Frequency of B10 cells from HC (n=10) and AAV patients (n=10) in remission. (**D**) Percentages of B10pro cells in HC (n=18), AAV patients in remission (n=15), AAV patients with active disease at the time of inclusion (n=6).

B cells was measured in 18 HCs, 15 remission and 6 active patients (figure 3D). In the HC group, the median percentage of IL-10-positive B cells was 9.2 (range 3.0-19.9), and this was not significantly different from the median level found in AAV patients in remission (median percentage 8.1, range 2.5-18.0) or patients with active disease (10.4%, range 4.9-21.8) (figure 3D). These data suggest that the overall capacity of B cells to produce IL-10 is not compromised in AAV patients.

B cell capacity to suppress monocyte activation is comparable in AAV patients and HCs

To investigate whether B cells from AAV patients can suppress pro-inflammatory cytokine production by other immune cells, we tested their ability to inhibit TNF-α production by monocytes activated with LPS. As patients often present with low numbers of lymphocytes, total CD19+ B cells were used in this suppression assay.

Sorted CD19+ cells were activated with CpG-ODN and stimulatory anti-CD40 antibody, and then co-cultured with autologous monocytes in a ratio of 1:1 for 24 h. In the final 4 h of the culture LPS was added to induce TNF-α production in monocytes. LPS-activated monocytes cultured in the absence of B cells were used as a positive control. For each individual, TNF-α expression in the positive control sample was set at 100% and the B cell-mediated suppression was calculated accordingly (figure 4A).

When monocytes were cultured in the presence of B cells that were not activated with CpG-ODN and anti-CD40, the median TNF-α production in monocytes was reduced by 38% in HCs and by 46.5% in GPA patients (figure 4B). This B cell-mediated suppressive effect was further enhanced if B cells had been activated with CpG-ODN and anti-CD40 prior to co-culture with monocytes, resulting in a median inhibition of 60% in HCs and 58% suppression in patients (figure 4B). These results demonstrate that B cells from AAV patients can suppress monocyte activation to a similar extent as those obtained from HCs, indicating that in AAV patients in remission B cell inhibitory function is not compromised.

DISCUSSION

AAV are autoimmune disorders in which both innate and adaptive immune mechanisms contribute to disease pathogenesis. B_{REG} cells have been shown to influence both arms of the immune system and have the potential to exert their modulatory effects on immune cells involved in the pathogenesis of AAV. In this study we characterized circulating B cell subpopulations, including two putative B_{REG} subsets, in patients with AAV and age- and sex-matched HCs. AAV patients during active disease had decreased frequencies of CD19+CD24^{high}CD38^{high} cells, while the proportions of CD19+CD24^{high}CD27+ cells were diminished independent of disease activity. However, no differences were found in the percentages of IL-10-producing B cells between AAV patients and HCs. In addition, B cells from AAV patients in remission inhibited monocyte activation to a similar extent as B cells obtained from HCs.

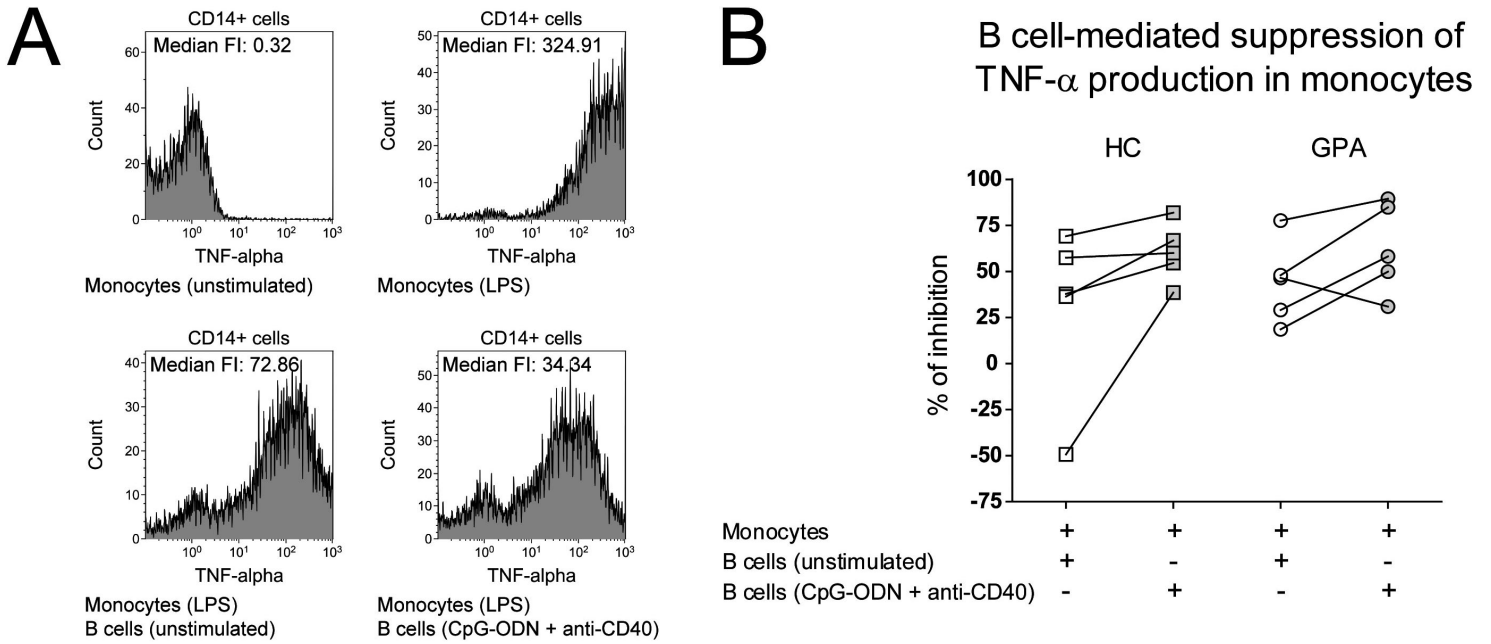


Figure 4. B cell-mediated suppression of TNF-α production in monocytes. In 5 HCs and 5 AAV patients in remission, B cell suppressive capacity was tested using LPS-activated monocytes as a responder population. Intracellular TNF-α expression in monocytes was measured by flow cytometry. (A) Representative histograms showing TNF-α expression in monocytes. Monocytes cultured alone without adding LPS were used as a negative control. Monocytes activated with LPS served as a positive control sample. Monocytes were co-cultured with either un-stimulated B cells or with B cells that were pre-activated with CpG-ODN and anti-CD40. (B) Data from 5 HCs and 5 GPA patients. For each donor, the median fluorescence intensity (FI) of TNF-α expression in the positive control condition was used as a reference sample and was normalized to 100%. Median FI values measured in other samples are expressed relative to the reference sample.

The data presented in this study show an overall altered distribution of transitional, naive and memory B cell subsets in AAV patients. In line with previous studies, we found a diminished frequency of memory B cells [21] independent of disease activity. In patients with active disease, the frequency of transitional B cells was also diminished when compared with HCs or patients in remission. Interestingly, the two putative B_{REG} subsets CD19+CD24^{high}CD38^{high} and CD19+CD24^{high}CD27+ were not overlapping and were contained within the transitional [22] and memory B cell compartments, respectively. Therefore the reduced proportion of circulating CD19+CD24^{high}CD38^{high} and CD19+CD24^{high}CD27+ cells that was observed in patients, is probably due to diminished transitional and memory subsets accordingly. Currently the reason for changes in B cell subset distribution is unclear. However, since B cells are known to be present in granulomatous lesions of AAV patients [23], we cannot exclude the possibility that the observed alterations in the peripheral blood occur due to B cell migration to the site of inflammation.

In humans, no consensus has been reached yet with regard to cell surface markers that identify a unique B_{REG} population and none of the currently proposed markers can exclusively distinguish IL-10-producing B cells from other B cell subsets. Also, a specific transcription factor, which could potentially be used to separate IL-10-producing B cells, has not been identified. Moreover, although studies have proposed the T cell Ig domain and mucin domain protein 1 (TIM-1) as a useful marker for delineation of B_{REGS} in mice [24], preliminary experiments from our group could not confirm this in humans (supplementary figure 1). Recently van de Veen *et al*. [25] used a whole-genome expression array to characterize markers differentially expressed by human IL-10-producing B cells compared with B cells that do not produce IL-10. Surprisingly, none of the previously reported markers was found to be specific for the IL-10-positive B cell compartment. The frequency of IL-10-positive B cells was found to be similar in CD19+CD24^{high}CD38^{high}, CD19+CD24^{int}CD38^{int}, and CD19+CD24^{high}CD38- subsets. Also the percentage of CD27+ B cells was equal between IL-10-positive and IL-10-negative B cells. This could explain why in our study the patients with decreased proportions of CD19+CD24^{high}CD38^{high} or CD19+CD24^{high}CD27+ cells, still displayed a normal frequency of IL-10-producing B cells. In contrast to our observations, a recent report by Wilde *et al*. [26] demonstrated a significantly decreased proportion of IL-10-positive B cells in the circulation of AAV patients. Additionally, in untreated patients in clinical remission, a negative association was found between IL-10-positive B cells and Th1 cells, which is in line with previous studies that suggested B_{REG} mediated suppression of the Th1 lineage [14, 18]. At present we cannot explain these discrepancies.

However, it should be taken into account that although IL-10-expressing B cells are considered the gold standard for distinguishing the B_{REG} subset, B_{REGS} also function through IL-10-independent mechanisms. These include production of TGF-β [27] as well as interaction with target cells via molecules expressed on the cell surface such as CD80 and CD86 [14]. Our data also strongly suggest that B cells to a large extent suppress monocyte activation in an IL-10-independent manner, as monocyte co-

culture with B cells without CpG-ODN and anti-CD40 activation resulted in significant inhibition of TNF-α production in monocytes. As B cells do not produce IL-10 without prior activation, this suggests that B cells inhibit TNF-α production through an alternative mechanism, although we have not dissected whether this inhibition is mediated via production of another soluble factor or through a cell-cell interaction.

In AAV, autoantibodies are considered central players in disease pathogenesis, as ANCA-mediated neutrophil and monocyte activation is considered a critical event leading to the blood vessel wall injury [1]. The use of B cell depletion therapy in AAV patients has not only proven clinically successful, but also has given more insight into the role of B cells in AAV. Rituximab-treated patients achieved clinical remission before the decline of circulating autoantibodies, suggesting that B cells also exert pathogenic effects in an autoantibody-independent fashion [5], although the underlying mechanisms are not yet clear. Also, patients benefit from B cell depletion therapy even when B_{REGS} are being depleted, suggesting that B cell mediated pro-inflammatory effects dominate over their regulatory function. Recent studies have identified IL-6 as an important candidate effector cytokine that mediates the pro-inflammatory effects of B cells [28]. In the experimental autoimmune encephalomyelitis (EAE) model, B cells are a major source of IL-6, and mice lacking IL-6-producing B cells had reduced disease severity associated with reduced IL-17 production by CD4+ T cells. Also, B cells isolated from MS patients secreted elevated amounts of IL-6 when compared with HCs. Interestingly, IL-6 production by B cells was normalized in the reconstituting B cell population after B cell depletion therapy. This suggests that the beneficial effects of B cell depletion therapy could be due in part to elimination of IL-6-producing B cells, which in turn leads to diminished Th17 responses. Since disturbances in T helper cell subsets [29-31], including increased Th17 cells [32-34], are well documented in AAV patients, future studies should determine not only the changes in the newly reconstituted B cell populations, but also the effects of B cell depletion therapy on the T cell compartment.

The (im)balance between regulatory and effector functions of B cells could contribute to the pathogenesis of autoimmune diseases, however, in AAV data on regulatory and effector B cells are limited. A recent study by Bunch *et al.* [35] reported that the percentage of circulating CD5+ B cells, which partially overlap the $CD19+CD24^{high}CD38^{high}$ phenotype, could be used as a biomarker to monitor disease activity and risk for relapse. A low percentage of circulating CD5+ B cells was shown to correlate with disease activity and shorter time to disease relapse. Also, patients after rituximab treatment whose B cells repopulated with low percentage of CD5+ B cells in conjunction with no or low immunosuppressive therapy were prone to relapse earlier than those who received high levels of maintenance immunosuppression. Although follow-up data were available from a limited number of patients, these data suggest that monitoring the B cell compartment could be informative in determining disease progression and activity, and possibly aid in predicting disease relapse.

The current study was designed as a cross-sectional study, and one of the main limitations is the lack of longitudinal data. Therefore the current data set cannot provide

information on alterations that possibly occur in the B cell compartment within individual patients during the course of the disease. Also, the number of patients that presented with active disease during this study (n=12) is limited and the consistency of findings in active disease should be verified in a larger cohort of patients. Although we confirmed that during remission the B cell suppressive capacity was not disturbed, further investigations are needed to determine whether this is also the case during active disease.

In summary, the putative B_{REG} subsets that have been proposed to harbour IL-10-competent B cells were diminished in the circulation of AAV patients. However, the ability of B cells to produce IL-10 and their capacity to suppress activation of other immune cells were not compromised in patients in clinical remission, implying that B_{REGS} are functional in AAV patients with quiescent disease. Longitudinal studies in remitting-relapsing disease are needed to evaluate whether B_{REGS} contribute to sustaining stable remission in AAV.

KEY MESSAGES

ANCA-associated vasculitis (AAV) patients have dysregulated homeostasis of circulating B cells.

B cell capacity to produce IL-10 and suppress monocyte activation is not impaired in AAV patients in remission.

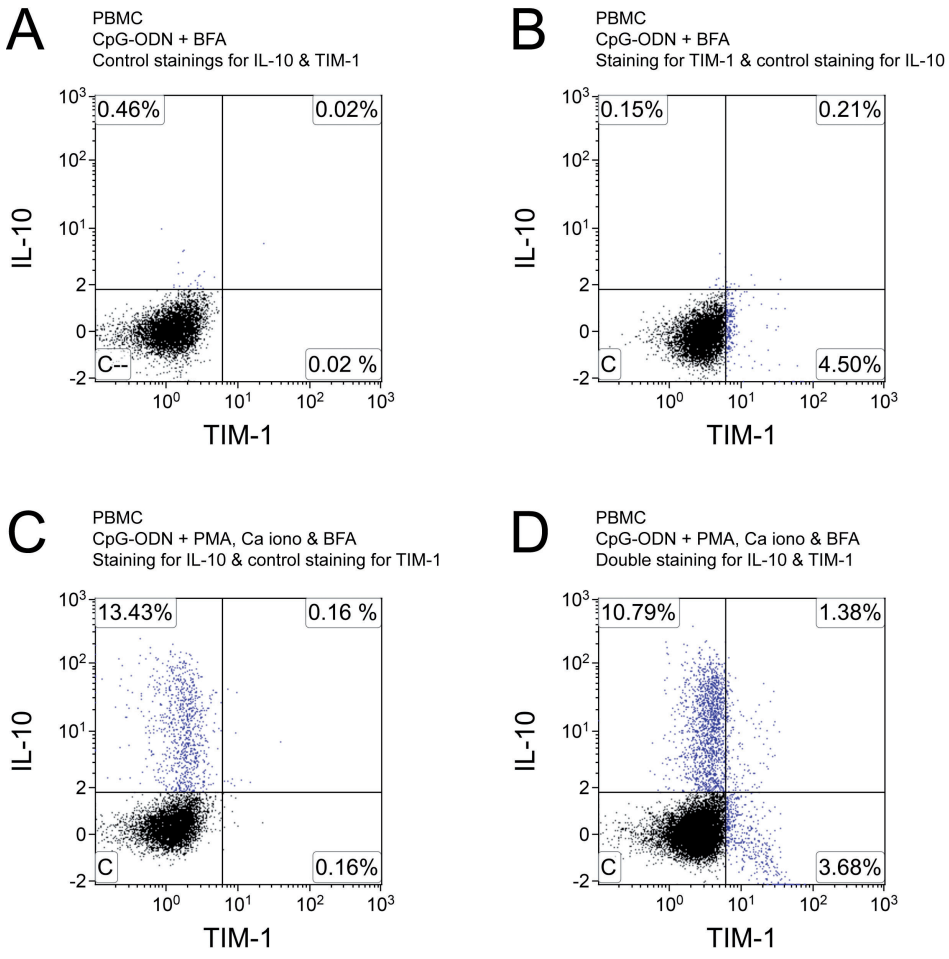
Future studies should investigate the clinical relevance of (im)balance between B cell pathogenic and suppressive functions in AAV.

ACKNOWLEDGEMENTS

We acknowledge Geert Mesander, Henk Moes, and Roelof Jan van der Lei from University Medical Center Groningen (UMCG) FACS facility for their technical assistance with cell sorting experiments. We would like to thank Prof. Pieter C. Limburg (Department of Laboratory Medicine, University of Groningen, UMCG) for his contribution in critical discussion of this work.

Research leading to these results has received funding from the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 261382, Dutch Arthritis foundation (Reumafonds project number 11-1-405 (A. R.)), and Groningen University Institute for Drug Exploration (GUIDE).

SUPPLEMENTARY DATA



Supplementary figure 1. Staining for IL-10 and TIM-1 in human B cells. Freshly isolated PBMCs from a healthy individual were stimulated with CpG-ODN for 3 days; in the final 5h of the experiment, cells were stimulated with or without PMA and calcium ionophore, and BFA was added to block protein secretion. At the end of the experiment, cells were stained for surface expression of B cell markers (CD19, CD22), TIM-1, and intracellular expression of IL-10. (**A-D**) In plots cells have been gated from the B cell gate. Plot **A** is a control staining for TIM-1 and IL-10; plot **B** shows staining for TIM-1 together with control staining for IL-10. In plot **C** the staining for IL-10 is shown with control staining for TIM-1; the plot **D** shows a double staining for TIM-1 and IL-10.

REFERENCES

1. Jennette JC, Falk RJ, Hu P, Xiao H: **Pathogenesis of antineutrophil cytoplasmic autoantibody-associated small-vessel vasculitis.** *Annu Rev Pathol* 2013, **8**:139-160.
2. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, Flores-Suarez LF, Gross WL, Guillevin L, Hagen EC, Hoffman GS, Jayne DR, Kallenberg CG, Lamprecht P, Langford CA, Luqmani RA, Mahr AD, Matteson EL, Merkel PA, Ozen S, Pusey CD, Rasmussen N, Rees AJ, Scott DG, Specks U, Stone JH, Takahashi K, Watts RA: **2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides.** *Arthritis Rheum* 2013, **65**(1):1-11.
3. Falk RJ, Gross WL, Guillevin L, Hoffman GS, Jayne DR, Jennette JC, Kallenberg CG, Luqmani R, Mahr AD, Matteson EL, Merkel PA, Specks U, Watts RA, American College of Rheumatology, American Society of Nephrology, European League Against Rheumatism: **Granulomatosis with polyangiitis (Wegener's): an alternative name for Wegener's granulomatosis.** *Arthritis Rheum* 2011, **63**(4):863-864.
4. Jones RB, Tervaert JW, Hauser T, Luqmani R, Morgan MD, Peh CA, Savage CO, Segelmark M, Tesar V, van Paassen P, Walsh D, Walsh M, Westman K, Jayne DR, European Vasculitis Study Group: **Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis.** *N Engl J Med* 2010, **363**(3):211-220.
5. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, Kallenberg CG, St Clair EW, Turkiewicz A, Tchao NK, Webber L, Ding L, Sejismundo LP, Mieras K, Weitzenkamp D, Ikle D, Seyfert-Margolis V, Mueller M, Brunetta P, Allen NB, Fervenza FC, Geetha D, Keogh KA, Kissin EY, Monach PA, Peikert T, Stegeman C, Ytterberg SR, Specks U, RAVE-ITN Research Group: **Rituximab versus cyclophosphamide for ANCA-associated vasculitis.** *N Engl J Med* 2010, **363**(3):221-232.
6. Cartin-Ceba R, Golbin JM, Keogh KA, Peikert T, Sanchez-Menendez M, Ytterberg SR, Fervenza FC, Specks U: **Rituximab for remission induction and maintenance in refractory granulomatosis with polyangiitis (Wegener's): ten-year experience at a single center.** *Arthritis Rheum* 2012, **64**(11):3770-3778.
7. Smith RM, Jones RB, Guerry MJ, Laurino S, Catapano F, Chaudhry A, Smith KG, Jayne DR: **Rituximab for remission maintenance in relapsing antineutrophil cytoplasmic antibody-associated vasculitis.** *Arthritis Rheum* 2012, **64**(11):3760-3769.
8. Lund FE, Randall TD: **Effector and regulatory B cells: modulators of CD4(+) T cell immunity.** *Nat Rev Immunol* 2010, **10**(4):236-247.
9. Mauri C: **Regulation of immunity and autoimmunity by B cells.** *Curr Opin Immunol* 2010, **22**(6):761-767.
10. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM: **B cells regulate autoimmunity by provision of IL-10.** *Nat Immunol* 2002, **3**(10):944-950.
11. Mauri C, Gray D, Mushtaq N, Londei M: **Prevention of arthritis by interleukin 10-producing B cells.** *J Exp Med* 2003, **197**(4):489-501.
12. Blair PA, Chavez-Rueda KA, Evans JG, Shlomchik MJ, Eddaoudi A, Isenberg DA, Ehrenstein MR, Mauri C: **Selective targeting of B cells with agonistic anti-CD40 is an efficacious strategy for the generation of induced regulatory T2-like B cells and for the suppression of lupus in MRL/lpr mice.** *J Immunol* 2009, **182**(6):3492-3502.
13. Carter NA, Vasconcellos R, Rosser EC, Tulone C, Munoz-Suano A, Kamanaka M, Ehrenstein MR, Flavell RA, Mauri C: **Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells.** *J Immunol* 2011, **186**(10):5569-5579.
14. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, Mauri C: **CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients.** *Immunity* 2010, **32**(1):129-140.
15. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, Szabolcs PM, Bernstein SH, Magro CM, Williams AD, Hall RP, St Clair EW, Tedder TF: **Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells.** *Blood* 2011, **117**(2):530-541.

16. Duddy M, Niino M, Adatia F, Hebert S, Freedman M, Atkins H, Kim HJ, Bar-Or A: **Distinct effector cytokine profiles of memory and naive human B cell subsets and implication in multiple sclerosis.** *J Immunol* 2007, **178**(10):6092-6099.
17. Knippenberg S, Peelen E, Smolders J, Thewissen M, Menheere P, Cohen Tervaert JW, Hupperts R, Damoiseaux J: **Reduction in IL-10 producing B cells (Breg) in multiple sclerosis is accompanied by a reduced naive/memory Breg ratio during a relapse but not in remission.** *J Neuroimmunol* 2011, **239**(1-2):80-86.
18. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, Mauri C: **CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation.** *Sci Transl Med* 2013, **5**(173):173ra23.
19. Jennette JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, Hagen EC, Hoffman GS, Hunder GG, Kallenberg CG: **Nomenclature of systemic vasculitides. Proposal of an international consensus conference.** *Arthritis Rheum* 1994, **37**(2):187-192.
20. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, Calabrese LH, Fries JF, Lie JT, Lightfoot RW,Jr: **The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis.** *Arthritis Rheum* 1990, **33**(8):1101-1107.
21. Tadema H, Abdulahad WH, Lepse N, Stegeman CA, Kallenberg CG, Heeringa P: **Bacterial DNA motifs trigger ANCA production in ANCA-associated vasculitis in remission.** *Rheumatology (Oxford)* 2011, **50**(4):689-696.
22. Marie-Cardine A, Divay F, Dutot I, Green A, Perdrix A, Boyer O, Contentin N, Tilly H, Tron F, Vannier JP, Jacquot S: **Transitional B cells in humans: characterization and insight from B lymphocyte reconstitution after hematopoietic stem cell transplantation.** *Clin Immunol* 2008, **127**(1):14-25.
23. Voswinkel J, Mueller A, Kraemer JA, Lamprecht P, Herlyn K, Holl-Ulrich K, Feller AC, Pitann S, Gause A, Gross WL: **B lymphocyte maturation in Wegener's granulomatosis: a comparative analysis of VH genes from endonasal lesions.** *Ann Rheum Dis* 2006, **65**(7):859-864.
24. Ding Q, Yeung M, Camirand G, Zeng Q, Akiba H, Yagita H, Chalasani G, Sayegh MH, Najafian N, Rothstein DM: **Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice.** *J Clin Invest* 2011, **121**(9):3645-3656.
25. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG, Ruckert B, Akdis CA, Akdis M: **IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses.** *J Allergy Clin Immunol* 2013, **131**(4):1204-1212.
26. Wilde B, Thewissen M, Damoiseaux J, Knippenberg S, Hilhorst M, van Paassen P, Witzke O, Cohen Tervaert JW: **Regulatory B cells in ANCA-associated vasculitis.** *Ann Rheum Dis* 2013, :.
27. Klinker MW, Lundy SK: **Multiple mechanisms of immune suppression by B lymphocytes.** *Mol Med* 2012, **18**:123-137.
28. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S, Fan B, O'Connor RA, Anderton SM, Bar-Or A, Fillatreau S, Gray D: **B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells.** *J Exp Med* 2012, **209**(5):1001-1010.
29. Abdulahad WH, van der Geld YM, Stegeman CA, Kallenberg CG: **Persistent expansion of CD4+ effector memory T cells in Wegener's granulomatosis.** *Kidney Int* 2006, **70**(5):938-947.
30. Abdulahad WH, Stegeman CA, van der Geld YM, Doornbos-van der Meer B, Limburg PC, Kallenberg CG: **Functional defect of circulating regulatory CD4+ T cells in patients with Wegener's granulomatosis in remission.** *Arthritis Rheum* 2007, **56**(6):2080-2091.
31. Free ME, Bunch DO, McGregor J, Jones BE, Berg EA, Hogan SL, Hu Y, Preston GA, Jennette JC, Falk RJ, Su MA: **ANCA-associated vasculitis patients have defective Treg function exacerbated by presence of a suppression-resistant effector population.** *Arthritis Rheum* 2013, :.
32. Abdulahad WH, Stegeman CA, Limburg PC, Kallenberg CG: **Skewed distribution of Th17 lymphocytes in patients with Wegener's granulomatosis in remission.** *Arthritis Rheum* 2008, **58**(7):2196-2205.
33. Nogueira E, Hamour S, Sawant D, Henderson S, Mansfield N, Chavele KM, Pusey CD, Salama AD: **Serum IL-17 and**

IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis. *Nephrol Dial Transplant* 2010, **25**(7):2209-2217.

34. Wilde B, Thewissen M, Damoiseaux J, Hilhorst M, van Paassen P, Witzke O, Cohen Tervaert JW: **Th17 expansion in granulomatosis with polyangiitis (Wegener's): the role of disease activity, immune regulation and therapy.** *Arthritis Res Ther* 2012, **14**(5):R227.
35. Bunch DO, McGregor JG, Khandoobhai NB, Aybar LT, Burkart ME, Hu Y, Hogan SL, Poulton CJ, Berg EA, Falk RJ, Nachman PH: **Decreased CD5(+) B cells in active ANCA vasculitis and relapse after rituximab.** *Clin J Am Soc Nephrol* 2013, **8**(3):382-391.

CHAPTER 6

THE HISTONE DEACETYLASE INHIBITOR TRICHOSTATIN A MODULATES CYTOKINE PRODUCTION IN HUMAN B CELLS

Nikola Lepse, Theo Bijma, Rutger A.F. Gjaltema,
Wayel H. Abdulahad and Peter Heeringa

WORK IN PROGRESS

ABSTRACT

Introduction

B cells can produce both anti- and pro-inflammatory cytokines; however, the pathways and mechanisms that regulate B cell cytokine production are ill-defined. In this study, we tested the hypothesis that histone/protein deacetylases (HDACs) regulate cytokine production in primary human B cells.

Materials and methods

Peripheral blood B cells from healthy individuals were stimulated with the Toll-like-receptor 9 (TLR9) ligand CpG-ODN to induce IL-10 production with or without the addition of the HDAC inhibitor trichostatin A (TSA). The effect of TSA treatment on IL-10, TNF-α, IL-6 and TGF- β1 mRNA and protein expression was determined by qRT-PCR and ELISA, respectively. Chromatin immunoprecipitation (ChIP) was used to assess the influence of TSA on the acetylation status of histone 3 (H3) and histone 4 (H4) of the *IL-10, TNF-α, IL-6* and *TGF- β1* promoters. To test whether NF-κB signaling regulates the expression of IL-10, TNF-α and IL-6 in human B cells, we tested the effect of NF-κB inhibitors SC-514 and Bay 11-7082 on production of these cytokines by flow cytometry.

Results

Treatment of B cells with TSA strongly inhibited CpG-ODN induced IL-10 and IL-6 production on the mRNA and protein level, while the production of TNF-α was increased. Treatment with TSA increased acetylation of H3 and H4 in the promoter regions of the *IL-10, TNF-α, IL-6* and *TGF- β1* genes. TSA-mediated inhibition of IL-10 and IL-6 production was further suppressed with the classical NF-κB pathway inhibitor SC-514, while TSA-enhanced TNF-α production could be partially suppressed only with Bay 11-7082, which inhibits both classical and alternative NF-κB signaling.

Conclusions

The HDAC inhibitor TSA differentially regulates cytokine production in human B cells. TSA-mediated inhibition of cytokine production cannot be explained by an altered histone acetylation status and likely occurs due to interference with the signaling pathways that regulate production of these cytokines.

INTRODUCTION

Under physiological circumstances B cells are critical players in the regulation of immune responses, providing protection against infection without causing overt damage to the host. This is accomplished through a finely regulated balance between pro-inflammatory and regulatory B cell functions that involves multiple B cell populations. B cells exert their effector functions through 3 major mechanisms – antibody production, antigen presentation and cytokine production [1]. In recent years, the cytokine producing function of B cells has gained considerable interest and it has been proposed that, based on their cytokine expression profile, B cells can be subdivided into regulatory and effector B cells [2]. B cells that produce the anti-inflammatory cytokine IL-10 are considered important negative regulators of the immune response [3] because these cells have been reported to suppress CD4+ T cell responses and monocyte activation in an IL-10-dependent manner [4]. In contrast, B cells competent of producing certain inflammatory cytokines are considered to promote Th17-mediated inflammatory responses. Studies both in multiple sclerosis patients and in murine experimental autoimmune encephalomyelitis (EAE) have demonstrated that IL-6 production by B cells contributes to IL-17 production by T cells [5]. In addition, it has been demonstrated that monocyte migration to sites of inflammation in tissue injury, after acute myocardial infarction, was mediated by B cells through production of the chemokine CCL7 [6]. Overall, the observations described above indicate that perturbations in B cell cytokines can contribute to disease pathogenesis, and therefore, modulation of B cell cytokine production is essential for proper B cell function. However, the mechanisms that control anti-inflammatory and pro-inflammatory cytokine production by B cells are poorly characterized, and the signaling pathways that favor B cell polarization towards a regulatory or effector phenotype are unclear.

Studies with CD4+ T helper cells (Th) have shown that commitment to a particular Th cell lineage is epigenetically regulated by means of DNA and histone methylation and histone acetylation [7]. These epigenetic modifications are regulated by methyltransferases, histone acetyltransferases and deacetylases and they control chromatin structure, DNA accessibility and gene expression. A class of drugs known as histone deacetylase (HDAC) inhibitors suppresses deacetylases, resulting in increased acetylation. Histone acetylation opens the chromatin structure, favoring gene transcription [7, 8]. Interestingly, HDAC inhibitors have been shown to promote the generation and function of regulatory T cells (T_{REGS}) *in vivo* [9]. Likewise, HDAC inhibitors promoted the function of human FoxP3+ T_{REGS} *in vitro* [10]. Whether regulatory and effector cytokine expression in B cells is also regulated by histone acetylation in a similar fashion is not known.

In the current study, we investigated the regulation of cytokine production in B cells and questioned the role of histone deacetylases in this process. To this end, we studied the effect of the HDAC inhibitor trichostatin A (TSA) on anti-inflammatory and pro-inflammatory cytokine production in B cells. Additionally, we examined the involvement of NF-κB signaling in the production of IL-10, TNF-α and IL-6.

MATERIALS AND METHODS

Cell isolation

Heparinized blood was collected from 9 healthy volunteers (4 males, 5 females) with mean age of 31.9 ± 6.8 years. Depending on the experiment, either peripheral blood mononuclear cells (PBMC) or sorted CD19+ cells were used. For PBMC isolation, the blood was diluted 1:1 in PBS (pH 7.4), overlaid on Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged for 20 minutes at 600 x *g*. PBMC were collected and washed twice with PBS. For the experiments where a purified B cell population was required, PBMC were labeled with anti-CD19-eFluor 450 (eBioscience, San Diego, CA, USA) and CD19+ cells were sorted with fluorescence activated cell sorting (FACS; MoFlo™, Beckman Coulter, Brea, CA, USA). The obtained population typically was > 95% pure. Cells were cultured in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 50 µg/mL gentamicin (GIBCO, Life Technologies, Grand Island, NY, USA) and 10% fetal calf serum (FCS; Lonza) in a flat bottom Costar plate (Corning Inc., Corning, NY, USA) at a concentration 1 x 10^6 cells/mL.

Cell stimulation and analysis of intracellular expression of IL-10, TNF-α and IL-6 by flow cytometry

Cells were stimulated for 72 hours with 500 ng/mL CpG-ODN 2006 (Hycult Biotech, Uden, the Netherlands), 50 µM SC-514 (InSolution™ IKK-2 Inhibitor; Calbiochem, EMD Millipore Corporation, Billerica, MA, USA), 1 µM Bay 11-7082 (Alexis Biochemicals Corp., San Diego, CA, USA), or the corresponding vehicle controls. Where indicated, 500 nM trichostatin A (TSA; Sigma-Aldrich, St. Louis, MO, USA) or the corresponding vehicle control was added during the last 24 hours of incubation. In the final 5 hours, 50 ng/mL of phorbol myristate acetate (PMA; Sigma-Aldrich), 2 mM calcium ionophore (Sigma-Aldrich) and 10 µg/mL brefeldin A (BFA; Sigma-Aldrich) were added to the culture. At the end of the experiment, cells were harvested and stained with anti-human CD19-eFluor 450 for 15 minutes in the dark. Further, the Fix&Perm kit (Invitrogen, Life Technologies, Grand Island, NY, USA) was used to fix and permeabilize the cells for intracellular cytokine detection. Then samples were incubated with antibodies against human IL-10-PE (BioLegend, San Diego, CA, USA), TNF-α Alexa Fluor® 488 (BD, Franklin Lakes, NJ, USA) and IL-6-APC (eBioscience). Cells treated with BFA only were used as a control sample for the intracellular staining to set the gate for data analysis. Samples were measured with a BD™ LSR-II flow cytometer (BD) and data were analyzed with Kaluza 1.2 Flow Analysis Software (Beckman Coulter).

Sorted B cells were cultured for 24 or 72 hours in the presence or absence of 500 ng/mL of CpG-ODN. If cells were stimulated only for 24 hours, TSA (500 nM) was added immediately at the beginning of the experiment, whereas for the 72 hour time point, TSA was added in the final 24 hours. At the end of the experiment, cells were harvested either for RNA isolation or chromatin immunoprecipitation (ChIP), and the culture supernatants were collected and stored at -20 °C until further use.

RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was isolated using the RNeasy® Plus Micro Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions. RNA was reverse-transcribed using random hexamer primers (Promega, Madison, WI, USA) and SuperScript® III Reverse Transcriptase (Invitrogen). Gene expression was measured with primer probe sets (all from Applied Biosystems, Foster City, CA, USA) specific for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs 99999905_m1), IL-10 (Hs00961622_m1), IL-6 (Hs00174131_m1), TGF- β1 (Hs00998133_m1) and TNF-α (Hs00174128_m1) with a TaqMan ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Gene expression was calculated relative to GAPDH as $2^{-\Delta CT}$, where ΔCT is $CT_{\text{gene of interest}} - CT_{GAPDH}$.

Enzyme linked immune-sorbent assay (ELISA) for IL-10, TNF-α and IL-6

In-house ELISAs were used to measure the levels of IL-10, TNF-α and IL-6 in the culture supernatants. 96-well Clear Flat Bottom Polystyrene High Bind Microplates (Corning Inc.) were coated with either 1 µg/mL rat anti-human IL-10 (BD), 2 µg/mL anti-human TNF-α (R&D Systems, Minneapolis, MN, USA) or 0.5 µg/mL anti-human IL-6 (R&D Systems). Plates were washed with washing buffer (0.025M Tris-HCl, 0.15M NaCl, 0.05% Tween-20) and blocked for 1 h with blocking buffer (PBS with 0.05% Tween-20 and 2% bovine serum albumin (BSA)). The supernatants were diluted in incubation buffer (PBS with 0.05% Tween-20 and 0.2% gelatin). Recombinant human (rh) IL-10 (BD), rhTNF-α (R&D Systems) or rhIL-6 (R&D Systems) with a known concentration were used to obtain standard curves. The bound cytokines were detected with biotynilated rat anti-human IL-10 (BD), goat anti-human TNF-α (R&D Systems), or goat anti-human IL-6 (R&D Systems). Further, samples were incubated with streptavidin conjugated to the horseradish peroxidase (HRP) (Sanquin, Amsterdam, the Netherlands). Tetramethylbenzidine dihydrochloride (TMB; Sigma-Aldrich) was used as substrate and the optical density was read at 450 nm using an Emax microplate reader (Molecular Devices, Silicon Valley, CA, USA).

Chromatin immunoprecipitation (ChIP) and analysis of histone 3 (H3) and histone 4 (H4) acetylation

Sorted B cells were stimulated with TSA (500 nM) or the corresponding amount of vehicle control (ethanol) for 24 hours at 37 °C with 5% CO_2. At the end of the experiment, cells were harvested and fixed with 1% formaldehyde solution. Then cells were washed with PBS, centrifuged for 5 min at 2000 rpm and the cell pellet was collected. Cells were lysed on ice with SDS lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.0, 10 mM EDTA) supplemented with proteinase inhibitor cocktail (Sigma-Aldrich), 1 mM PMSF and 20mM Na-butyrate. Chromatin was fragmented with a Bioruptor® sonicator (Diagenode, Searing, Belgium) and cleared by centrifugation. Afterwards the chromatin was diluted 10 times in RIPA buffer (0.1% SDS, 0.1% Na-deoxycholate, 1% Triton-X100, 1 mM EDTA, 10 mM Tris-HCl pH 7.5, 140 mM NaCl, 0.5 mM EGTA) supplemented with a proteinase inhibitor cocktail (Sigma Aldrich), 1 mM PMSF and 20 mM Na-butyrate. Protein-A Dynabeads (Life technologies)

were coated with 4 µg antibodies against rabbit IgG (rIgG; Abcam, Cambridge, the United Kingdom), acetylated H3 (acH3) or acH4 (both from Merck Millipore, Billerica, MA, USA) and incubated with diluted chromatin of 200,000 cells per ChIP reaction overnight at 4 °C. The following day the beads were washed 3 times with PBS and eluted (1% SDS, 100 mM $NaHCO_3$). The eluted DNA was decross-linked and treated with RNase and Proteinase K (both from Roche, Basel, Switzerland). DNA was purified with a PCR purification kit (Qiagen) following the manufacturer's protocol. ChIP DNA fragments were quantified by qRT-PCR using Sybr Green (Roche) and primers for the proximal promoters of IL6 (Fw: CAAGACATGCCAAAGTGCTG, Rev: GAGGCTAGCGCTAAGAAGCA), IL10 (Fw: GCAATTTGTCCACGTCACTG, Rev: AAGTTGATTTCCTGGGGAGA), TNF-α (Fw: GGGAGTGTGAGGGGTATCCT, Rev: GCACCTTCTGTCTCGGTTTC) and TGF-β1 (Fw: GTCTCATCCCCCGGATTAAG, Rev: AAGCGGGTGATCCAGATG). Data is represented as fold enrichment relative to control rIgG (2^ΔΔCt).

Statistical analysis

Data are presented as mean values ± SEM, unless stated otherwise. Due to the limited number of donors, data was assumed to have non-Gaussian distribution. A Friedman test was used for intra-individual comparison with more than 2 groups. If a significant difference was found, further testing was performed using the Wilcoxon matched pairs test. P value < 0.05 was considered statistically significant. *P<0.05; **P<0.001; ***P<0.0001.

RESULTS

IL-10 and TNF-α are predominantly produced by distinct B cell subsets

Previously, we and others have demonstrated that B cells are competent of producing IL-10 [4, 11]. Less is known about effector cytokine production by B cells. To gain more insight into the normal distribution and balance between regulatory and effector B cells, we first characterized the frequency of IL-10 and TNF-α producing B cells in healthy individuals (figure 1A and 1B).

As activation through Toll-like receptor 9 (TLR9) is required to induce robust IL-10 production in B cells [4], separate PBMC samples were stimulated with the TLR9 ligand CpG-ODN. Upon stimulation with PMA and calcium ionophore (PMA/Ca iono) in the absence of CpG-ODN, the percentage of IL-10+ B cells was low (median frequency 0.36% (range 0.0-1.27)) whereas 22.5% (13.4-35.0) of B cells produced TNF-α. Upon PMA/Ca iono stimulation in the presence of CpG-ODN, IL-10 production could be detected in 9.3% (4.1-19.9) of B cells, while the percentage of TNF-α-producing B cells significantly decreased (median percentage 9.5% (4.9-18.2)) (figure 1B). Interestingly, a population of B cells double-positive for IL-10 and TNF-α could also be detected in all individuals (figure 1B).

TSA modulates anti- and pro-inflammatory cytokine production in B cells

We next questioned whether IL-10 and TNF-α expression in B cells is regulated by histone deacetylases. To this end, we tested the effect of the HDAC inhibitor TSA on the

production of IL-10 and TNF-α (figure 1C). We observed that treatment with TSA had a marked effect on cytokine production by B cells. Whereas TSA completely abolished IL-10 expression, it simultaneously promoted the production of TNF-α (figure 1C).

TSA alters cytokine expression in B cells on mRNA level

To exclude the possibility that TSA-induced modulation of cytokine production in B cells occurs indirectly due to effects from other cells present in the PBMC culture, B cells were purified by FACS and B cell cytokine mRNA and protein levels upon treatment with CpG-ODN and TSA were measured.

Treatment with CpG-ODN significantly increased mRNA expression of IL-10, TNF-α and IL-6, but inhibited transcription of TGF- β1 (figure 2A). Treatment with TSA strongly inhibited the basal, as well as the CpG-ODN-induced mRNA expression of IL-10 and IL-6. TSA also had an inhibitory effect on mRNA expression of TGF- β1. In contrast, TSA increased transcription of TNF-α (figure 2A).

On the protein level, CpG-ODN significantly increased production of IL-10, TNF-α and IL-6 (figure 2B), whereas TSA strongly inhibited production of IL-10 and IL-6. While there was a trend for increased TNF-α production upon stimulation with TSA (P=0.08), combined treatment with CpG-ODN and TSA induced significantly more TNF-α production than treatment with CpG-ODN alone (figure 2B).

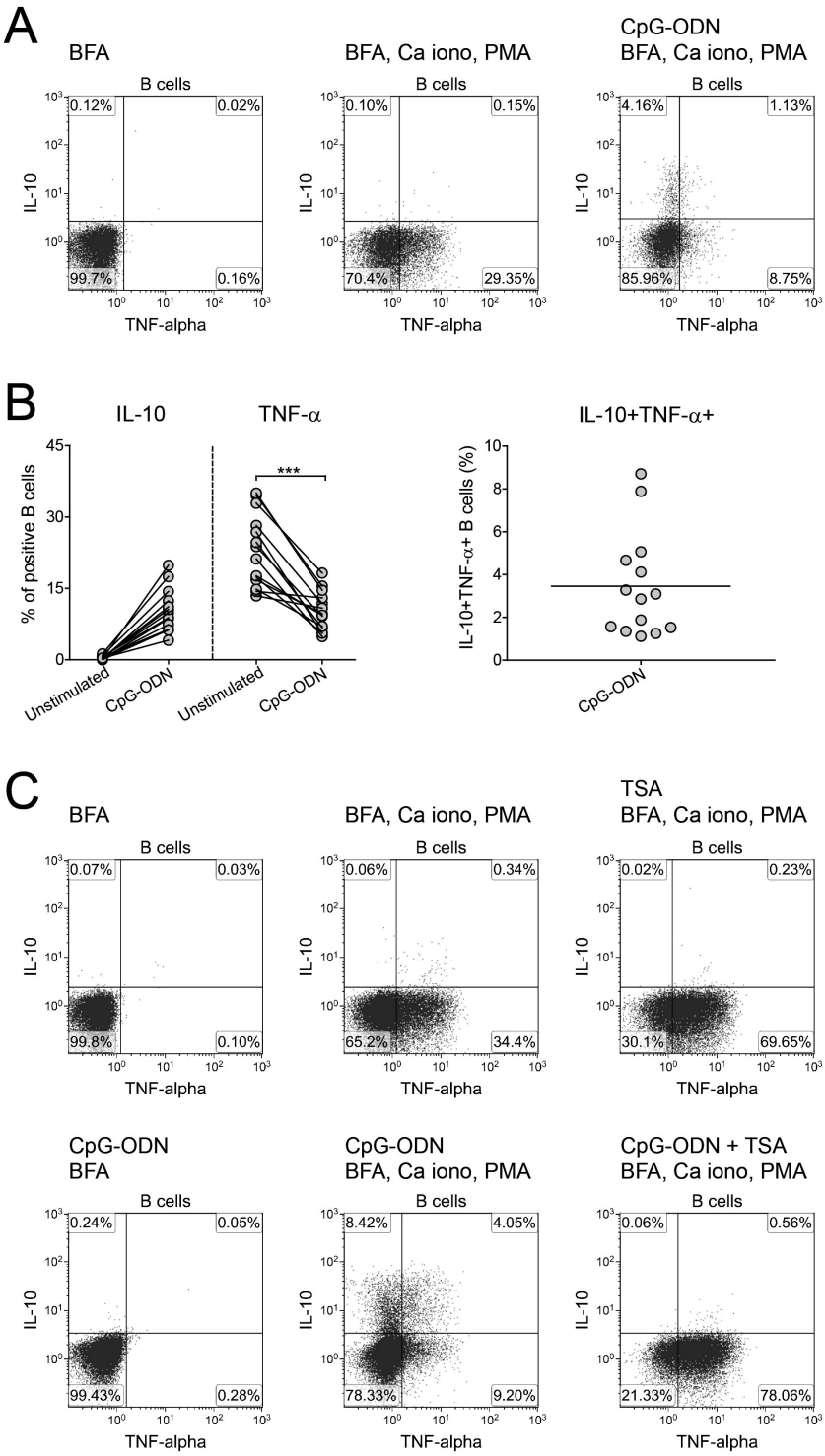
TSA increases histone 3 (H3) and histone 4 (H4) acetylation of *IL-10*, *TNF-α*, *IL-6* and *TGF-β1* genes

We further evaluated whether TSA-mediated alterations in expression of IL-10, TNF-α, IL-6 and TGF- β1 are associated with changes in acetylation of histones located in the proximal promoters of these genes. We analyzed changes in total lysine acetylation of H3 and H4, which are permissive epigenetic marks and associated with gene transcription. Interestingly, we found that TSA increased acetylation of H3 and H4 of all the genes of interest (figure 3), including IL-10 and IL-6, which were profoundly inhibited by TSA on mRNA and protein level (figure 2).

IL-10, TNF-α and IL-6 production in B cells is partially mediated through NF-κB

Because TNF-α and IL-6 are known to be NF-κB responsive genes [12], we examined the involvement of NF-κB signaling in the production of IL-10, TNF-α and IL-6 employing two inhibitors that are known to interfere with NF-κB signaling. SC-514 is an inhibitor which specifically inhibits the classical NF-κB pathway, whereas Bay 11-7082 non-specifically inhibits both the classical and the non-classical NF-κB pathway.

Inhibition of the classical NF-κB pathway with SC-514 significantly reduced production of IL-10, TNF-α and IL-6. Interestingly, treatment with Bay 11-7082 did not have a significant effect on IL-10 production, while it significantly suppressed the production of TNF-α and had an even stronger suppressive effect on IL-6 production (figure 4).


A
BFA
B cells
0.12%
0.02%
99.7%
0.16%
IL-10
TNF-alpha
BFA, Ca iono, PMA
B cells
0.10%
0.15%
70.4%
29.35%
IL-10
TNF-alpha
CpG-ODN
BFA, Ca iono, PMA
B cells
4.16%
1.13%
85.96%
8.75%
IL-10
TNF-alpha
B
IL-10
TNF-α
% of positive B cells
45
30
15
0
***
Unstimulated
CpG-ODN
Unstimulated
CpG-ODN
IL-10+TNF-α+
IL-10+TNF-α+ B cells (%)
10
8
6
4
2
0
CpG-ODN
C
BFA
B cells
0.07%
0.03%
99.8%
0.10%
IL-10
TNF-alpha
BFA, Ca iono, PMA
B cells
0.06%
0.34%
65.2%
34.4%
IL-10
TNF-alpha
TSA
BFA, Ca iono, PMA
B cells
0.02%
0.23%
30.1%
69.65%
IL-10
TNF-alpha
CpG-ODN
BFA
B cells
0.24%
0.05%
99.43%
0.28%
IL-10
TNF-alpha
CpG-ODN
BFA, Ca iono, PMA
B cells
8.42%
4.05%
78.33%
9.20%
IL-10
TNF-alpha
CpG-ODN + TSA
BFA, Ca iono, PMA
B cells
0.06%
0.56%
21.33%
78.06%
IL-10
TNF-alpha

◀ **Figure 1. TSA modulates IL-10 and TNF-α production in B cells.** (**A**) Representative flow cytometry dot plots from 1 healthy individual demonstrating IL-10 and TNF-α expression in B cells. (**B**) Frequencies of IL-10 single positive (left panel), TNF-α single positive (middle panel) with or without CpG-ODN stimulation and IL-10/TNF-α double positive B cells with CpG-ODN stimulation in cells obtained from 14 healthy individuals. The data, showing IL-10 single positive B cells upon stimulation with CpG-ODN, has been reported previously in Lepse *et al*. [11]. (**C**) Representative dot plots from 3 independent experiments showing the effect of TSA on IL-10 and TNF-α production in primary human B cells. Cells were cultured in the presence or absence of CpG-ODN (500 ng/ml) for 3 days. TSA (500 nM) was added during the final 24 hours of the culture where indicated. In the final 5 hours, cells were re-stimulated with PMA and calcium ionophore.

Bay 11-7082 but not SC-514 partially inhibits TSA-induced TNF-α production

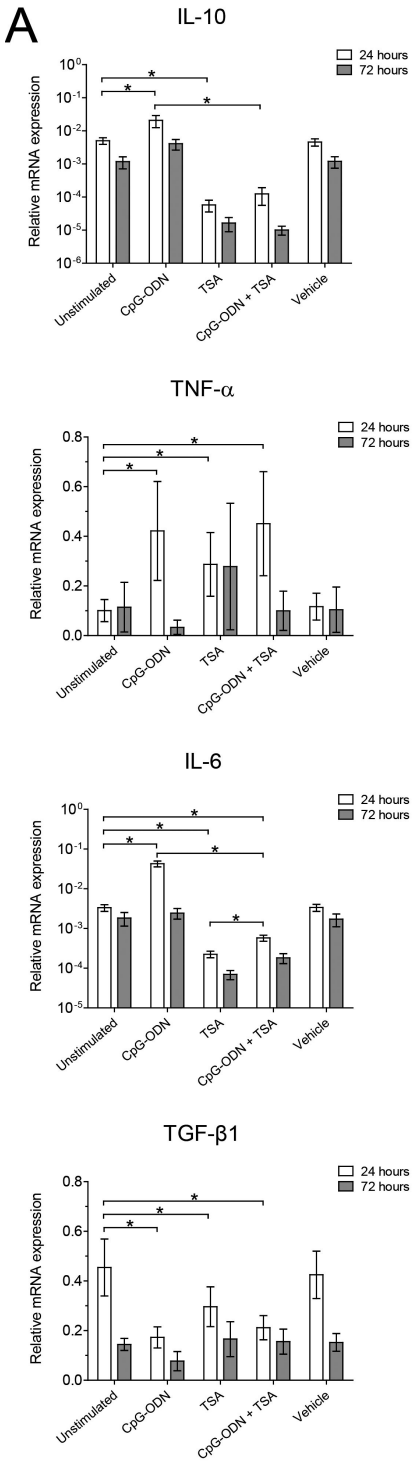
We further questioned whether the observed modulatory effects of TSA on B cell cytokine production are mediated through the NF-κB pathway. TSA-induced inhibition of IL-10 and IL-6 could be further suppressed by the addition of SC-514. In contrast, TSA-induced TNF-α production was not affected by inhibition of the classical NF-κB pathway, but it could be partially inhibited with Bay 11-7082 (figure 5).

DISCUSSION

In the past decade, B cell depletion therapy with anti-CD20 antibodies is being used as a treatment for patients with autoimmune diseases, such as rheumatoid arthritis (RA) and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) [13-15]. In these diseases, patients often achieve clinical remission before the clearance of the autoantibodies from the circulation, indicating that antibody-independent functions of B cells contribute to the beneficial effects of B cell depletion therapy. A number of studies have investigated the role of IL-10-producing B cells in the regulation of immune responses [3]. However, little is known about the pro-inflammatory cytokines produced by B cells and their impact on the immune system. Furthermore, the signaling pathways which skew B cells towards production of either anti- or pro-inflammatory cytokines are not well-defined. In this study, we show that the HDAC inhibitor TSA modulates pro- and anti-inflammatory cytokine expression in primary human B cells on the transcriptional and protein level. We found that TSA strongly down-regulates the expression of IL-10 and IL-6 but up-regulates the production of TNF-α.

Besides histones, also non-histone proteins can be post-translationally modified by acetylation. Thus, inhibition of HDACs can affect various proteins in the signal transduction pathways involved in cytokine production, including transcription factors. TSA modulated cytokine expression in B cells as observed in our study probably occurs either due to an altered acetylation state of histones or due to interference with these signaling pathways.

In order to test whether the observed alterations in cytokine expression are associated with an altered acetylation status of histones, we analyzed the extent of lysine acetylation of histone 3 (H3) and histone 4 (H4) of the *IL-10*, *TNF-α*, *IL-6* and *TGF-β1* genes in B cells upon treatment with TSA. We found that TSA increased H3


A
IL-10
Relative mRNA expression
24 hours
72 hours
Unstimulated
CpG-ODN
TSA
CpG-ODN + TSA
Vehicle
B
IL-10
pg/mL
TNF-α
P=0.08
CpG-ODN & TSA
IL-6
TGF-β1

◀ **Figure 2. TSA alters cytokine mRNA and protein expression.** Sorted CD19+ cells were cultured in the presence or absence of CpG-ODN (500 ng/mL) and/or TSA (500 nM). (**A**) IL-10, TNF-α, IL-6 and TGF-β1 mRNA expression as quantified by RT-qPCR and expressed relative to GAPDH. (**B**) IL-10, TNF-α and IL-6 protein levels as measured in the supernatants by ELISA. The graphs represent the mean values ± SEM. Six healthy individuals were included in the 24h time point and 3 healthy individuals were included in the 72h time point.

and H4 acetylation in the proximal promoter regions of the *IL-10*, *TNF-α*, *IL-6* and *TGF- β1* genes. Because acetylation of H3 and H4 are typically associated with gene transcription [7], these data do not explain the TSA-mediated suppression of IL-10 and IL-6. Alternatively, TSA might affect proteins involved in the signal transduction pathways responsible for B cell cytokine production. It is well known that protein acetylation can regulate the activity of various transcription factors by modifying protein stability and interfering with protein-protein interactions [16]. For example, acetylation of the transcription factor FoxP3 in T_{REGS} increases its capacity to bind to DNA [9], which explains the enhanced suppressive activity of T_{REGS} upon treatment with HDAC inhibitors [9, 10]. The activity of NF-κB is also known to be regulated by acetylation. However, depending on the site where the acetyl group is added, acetylation of p65 subunit of NF-κB can have opposing effects on its DNA binding and transcriptional activity. For example, acetylation of lysine 221 of p65 increases NF-κB binding to DNA, while acetylation of lysines 122 and 123 decreases its affinity for DNA [16, 17].

In this study, we also questioned whether production of IL-10, TNF-α and IL-6 is mediated through NF-κB. Inhibition of IKK- β which is required for activation of the classical NF-κB pathway (figure 6) strongly diminished production of IL-10. At the same time, IL-10 could not be inhibited with Bay 11-7082, which non-specifically interferes with both the classical and non-classical NF-kB pathways by inhibiting IκBα, IKK-α and IKK- β (figure 6). Because IKK- β is also involved in other signaling pathways [18], we cannot exclude that IL-10 production is mediated through an NF-κB-independent mechanism. In order to conclusively prove or disprove that IL-10 production is mediated via the canonical NF-κB pathway, future experiments should determine if activation of p65 is required for IL-10 production in B cells. Moreover, blockade of IKK- β was recently shown to inhibit signaling of signal transducer and activator of transcription (STAT) 3 and mammalian target of rapamycin (mTOR) in T cells, leading to a decreased production of IL-10 [19]. These pathways also might play a role in IL-10 production in B cells.

Blockade of IKK- β with inhibitor SC-514 partially reduced the production of TNF-α and IL-6 and these cytokines also could be suppressed with Bay 11-7082, suggesting that the production of these cytokines in B cells is NF-κB-dependent. However, both inhibitors suppressed TNF-α and IL-6 only partially; therefore, additional signaling pathways (e.g., p38 MAPK) might be involved as well [20].

Both blockade of IKK- β and treatment with TSA inhibited IL-10 production, suggesting that TSA interferes with the signaling pathway mediated by IKK- β. This notion is supported by the observation that TSA-mediated inhibition of IL-10 and IL-6 could be

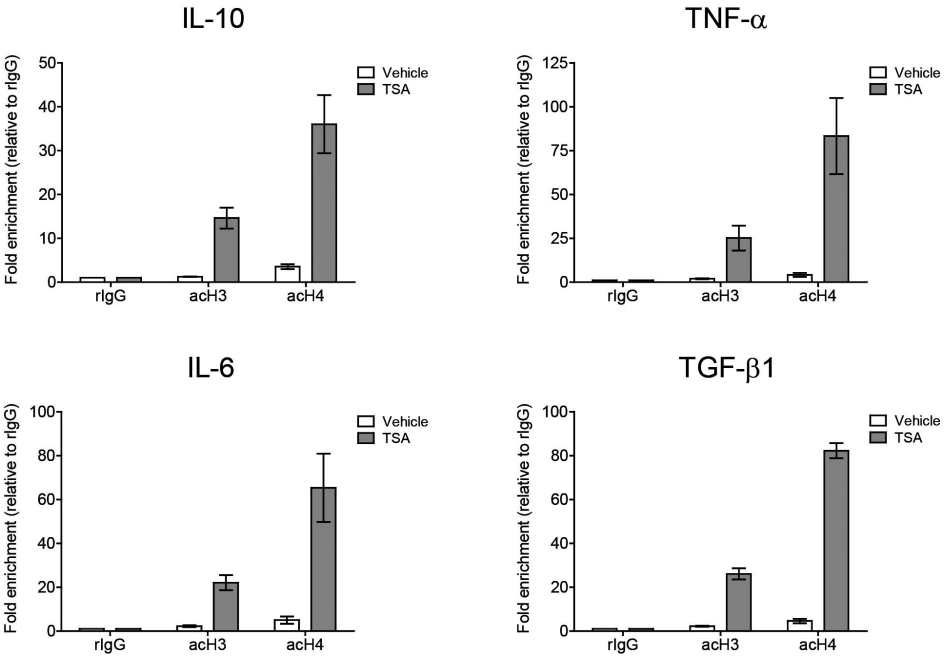


Figure 3. TSA increases acetylation of H3 and H4 of the *IL-10*, *TNF*-α, *IL-6* and *TGF*-β*1* genes. Sorted CD19+ cells were treated with 500 nM TSA or the vehicle control for 24 hours. The acetylation status of histones H3 and H4 was analyzed by ChIP and RT-qPCR. Rabbit IgG was used as negative control. The graphs represent data from 3 healthy donors.

further suppressed by addition of the IKK- β inhibitor SC-514. However, to determine whether TSA inhibits signaling of NF-κB or other IKK- β mediated pathway, e.g., STAT, it is necessary to monitor the activity of the signaling proteins involved in the respective pathways, for example, by determining the phosphorylation status of STAT3 and p65.

HDAC inhibitors, which are primarily being used as anti-cancer drugs, are also considered promising agents for the treatment of autoimmune diseases, organ transplant rejection and graft-versus-host disease (GVHD) due to their inhibitory effects on T cells, monocytes, macrophages and dendritic cells [8, 10]. Recently it was shown that treatment with valproic acid (VPA), which is a class I HDAC inhibitor, delays B cell differentiation into plasmablasts [21]. In this context, our finding that TSA up-regulates TNF-α in B cells contradicts with the anti-inflammatory effects of HDAC inhibitors observed on other immune cells. Thus, more studies are needed to elucidate the functional significance of TNF-α up-regulation in B cells. Interestingly, epilepsy patients treated with VPA have been reported to display significantly decreased IL-10 serum levels [22]. In the same study, there was a trend for decreased IL-6 serum levels but this did not reach statistical significance whereas serum levels of TNF-α were not significantly affected by VPA treatment [22]. This might be due to the higher selectivity of VPA towards class I HDACs when compared to TSA which affects both class I and class II HDACs [23].

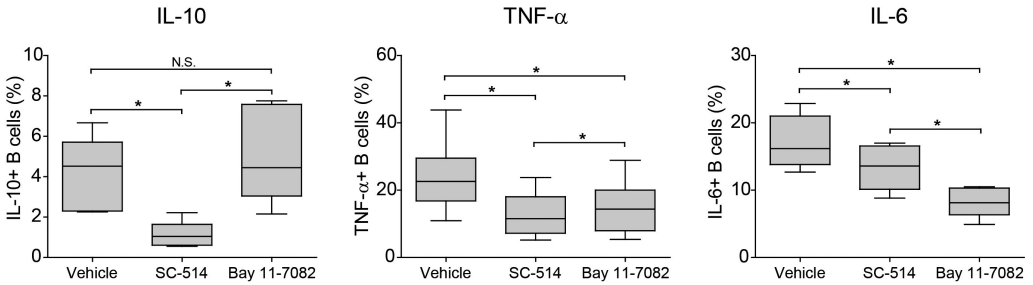


Figure 4. The effect of SC-514 and Bay 11-7082 on IL-10, TNF-α and IL-6 production in B cells. PBMC were treated with 50 μM SC-514, 1 μM Bay 11-7082, or the corresponding vehicle control (DMSO) for 72 hours in the presence (for IL-10 detection) or absence (for TNF-α and IL-6 detection) of CpG-ODN (500 ng/mL). In the final 5 hours, cells were re-stimulated with PMA/Calcium ionophore in the presence of BFA. Intracellular cytokine expression was measured by flow cytometry and expressed as the proportion of positive cells. The box and whisker plots represent data from 6 healthy individuals. NS: not significant.

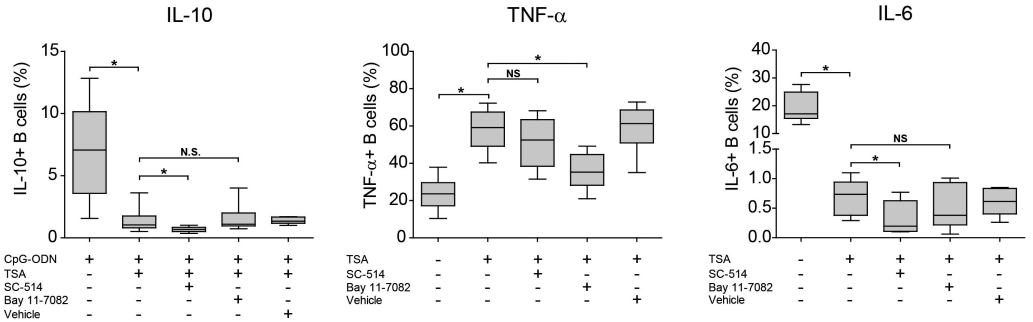


Figure 5. SC-514 enhances TSA-mediated IL-10 and IL-6 inhibition, whereas Bay 11-7082 suppresses TSA-induced TNF-α production. PBMC were treated with 50 μM SC-514, 1 μM Bay 11-7082 or the corresponding vehicle control for 72 hours in the presence or absence of CpG-ODN (500 ng/mL). TSA (500 nM) was added to the cultures in the last 24 hours. In the final 5 hours of the experiment, cells were re-stimulated with PMA/Calcium ionophore in the presence of BFA. Intracellular cytokine expression was measured by flow cytometry and expressed as the proportion of positive cells. The box and whisker plots represent data from 6 healthy individuals. NS: not significant.

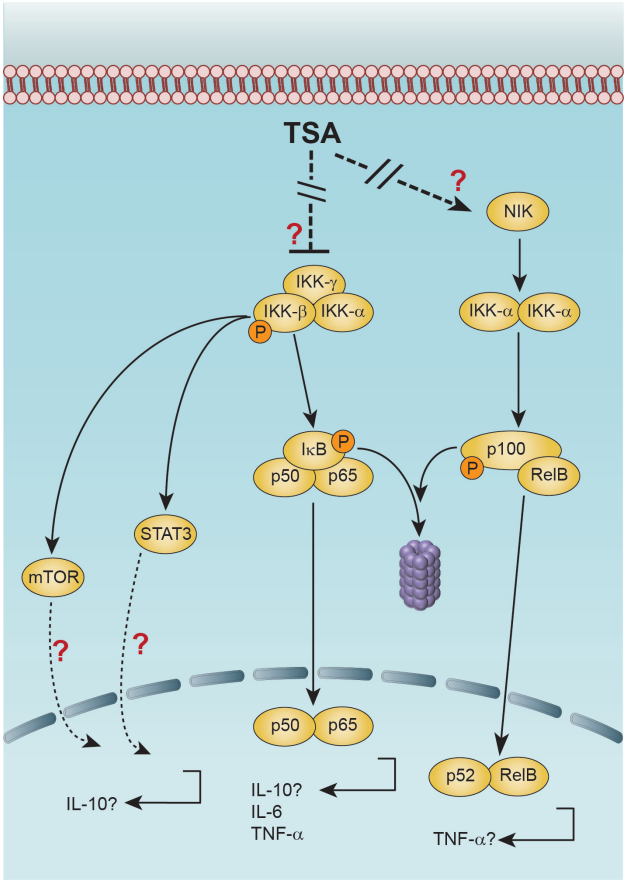


Figure 6. Schematic representation of the putative signal transduction pathways involved in IL-10, IL-6 and TNF-α production in B cells. Upon cell activation, IKK-β is phosphorylated which in turn phosphorylates IκB leading to its degradation. Then the p50/p65 dimer can translocate to the nucleus and induce transcription of NF-κB dependent genes, such as IL-6 and TNF-α, and possibly IL-10 as blockade of IKK-β resulted in a strong inhibition of IL-10 production. Because IKK-β also mediates signaling through other pathways (e.g. mTOR and STAT3) these pathways may be involved in IL-10 production as well. As inhibition of IKK-β with SC-514 enhanced TSA-mediated suppression of IL-10 and IL-6, we propose that TSA directly or indirectly interferes with IKK-β mediated signaling. On the other hand, TSA strongly enhanced TNF-α production which could not be suppressed by inhibition of IKK-β. As treatment with Bay 11-7082 reduced TSA-mediated TNF-α production, it suggests that TSA has a stimulatory effect on the alternative NF-κB pathway.

In conclusion, the data presented here demonstrate that the HDAC inhibitor TSA alters anti-inflammatory and pro-inflammatory cytokine expression in human B cells. Further studies are needed to determine the exact intracellular pathways that are modulated by TSA. Understanding the underlying pathways that regulate this phenomenon might give clues to approaches that modulate cytokine expression in B cells in a targeted fashion.

REFERENCES

1. DiLillo DJ, Horikawa M, Tedder TF: **B-lymphocyte effector functions in health and disease.** *Immunol Res* 2011, **49**(1-3):281-292.
2. Lund FE, Randall TD: **Effector and regulatory B cells: modulators of CD4(+) T cell immunity.** *Nat Rev Immunol* 2010, **10**(4):236-247.
3. Kalampokis I, Yoshizaki A, Tedder TF: **IL-10-producing regulatory B cells (B10 cells) in autoimmune disease.** *Arthritis Res Ther* 2013, **15 Suppl 1**:S1.
4. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, Szabolcs PM, Bernstein SH, Magro CM, Williams AD, Hall RP, St Clair EW, Tedder TF: **Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells.** *Blood* 2011, **117**(2):530-541.
5. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S, Fan B, O'Connor RA, Anderton SM, Bar-Or A, Fillatreau S, Gray D: **B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells.** *J Exp Med* 2012, **209**(5):1001-1010.
6. Zouggari Y, Ait-Oufella H, Bonnin P, Simon T, Sage AP, Guerin C, Vilar J, Caligiuri G, Tsiantoulas D, Laurans L, Dumeau E, Kotti S, Bruneval P, Charo IF, Binder CJ, Danchin N, Tedgui A, Tedder TF, Silvestre JS, Mallat Z: **B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction.** *Nat Med* 2013, **19**(10):1273-1280.
7. Wilson CB, Rowell E, Sekimata M: **Epigenetic control of T-helper-cell differentiation.** *Nat Rev Immunol* 2009, **9**(2):91-105.
8. Roger T, Lugrin J, Le Roy D, Goy G, Mombelli M, Koessler T, Ding XC, Chanson AL, Reymond MK, Miconnet I, Schrenzel J, Francois P, Calandra T: **Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection.** *Blood* 2011, **117**(4):1205-1217.
9. Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, Li B, Turka LA, Olson EN, Greene MI, Wells AD, Hancock WW: **Deacetylase inhibition promotes the generation and function of regulatory T cells.** *Nat Med* 2007, **13**(11):1299-1307.
10. Akimova T, Ge G, Golovina T, Mikheeva T, Wang L, Riley JL, Hancock WW: **Histone/protein deacetylase inhibitors increase suppressive functions of human FOXP3+ Tregs.** *Clin Immunol* 2010, **136**(3):348-363.
11. Lepse N, Abdulahad WH, Rutgers A, Kallenberg CG, Stegeman CA, Heeringa P: **Altered B cell balance, but unaffected B cell capacity to limit monocyte activation in anti-neutrophil cytoplasmic antibody-associated vasculitis in remission.** *Rheumatology (Oxford)* 2014, :.
12. Ahn KS, Aggarwal BB: **Transcription factor NF-kappaB: a sensor for smoke and stress signals.** *Ann N Y Acad Sci* 2005, **1056**:218-233.
13. van Vollenhoven RF, Emery P, Bingham CO,3rd, Keystone EC, Fleischmann RM, Furst DE, Tyson N, Collinson N, Lehane PB: **Long-term safety of rituximab in rheumatoid arthritis: 9.5-year follow-up of the global clinical trial programme with a focus on adverse events of interest in RA patients.** *Ann Rheum Dis* 2013, **72**(9):1496-1502.
14. Jones RB, Tervaert JW, Hauser T, Luqmani R, Morgan MD, Peh CA, Savage CO, Segelmark M, Tesar V, van Paassen P, Walsh D, Walsh M, Westman K, Jayne DR, European Vasculitis Study Group: **Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis.** *N Engl J Med* 2010, **363**(3):211-220.
15. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, Kallenberg CG, St Clair EW, Turkiewicz A, Tchao NK, Webber L, Ding L, Sejismundo LP, Mieras K, Weitzenkamp D, Ikle D, Seyfert-Margolis V, Mueller M, Brunetta P, Allen NB, Fervenza FC, Geetha D, Keogh KA, Kissin EY, Monach PA, Peikert T, Stegeman C, Ytterberg SR, Specks U, RAVE-ITN Research Group: **Rituximab versus cyclophosphamide for ANCA-associated vasculitis.** *N Engl J Med* 2010, **363**(3):221-232.
16. Glozak MA, Sengupta N, Zhang X, Seto E: **Acetylation and deacetylation of non-histone proteins.** *Gene* 2005, **363**:15-23.
17. Ghizzoni M, Haisma HJ, Maarsingh H, Dekker FJ: **Histone acetyltransferases are crucial regulators in NF-kappaB mediated inflammation.** *Drug Discov Today* 2011, **16**(11-12):504-511.
18. Liu F, Xia Y, Parker AS, Verma IM: **IKK biology.** *Immunol Rev* 2012, **246**(1):239-253.

19. Nishioka C, Ikezoe T, Yang J, Udaka K, Yokoyama A: **The combination of IkappaB kinase beta inhibitor and everolimus modulates expression of interleukin-10 in human T-cell lymphotropic virus type-1-infected T cells.** *Immunology* 2013, **138**(3):216-227.
20. Agrawal S, Gollapudi S, Su H, Gupta S: **Leptin activates human B cells to secrete TNF-alpha, IL-6, and IL-10 via JAK2/STAT3 and p38MAPK/ERK1/2 signaling pathway.** *J Clin Immunol* 2011, **31**(3):472-478.
21. Kienzler AK, Rizzi M, Reith M, Nutt SL, Eibel H: **Inhibition of human B-cell development into plasmablasts by histone deacetylase inhibitor valproic acid.** *J Allergy Clin Immunol* 2013, **131**(6):1695-1699.
22. Sonmez FM, Serin HM, Alver A, Aliyazicioglu R, Cansu A, Can G, Zaman D: **Blood levels of cytokines in children with idiopathic partial and generalized epilepsy.** *Seizure* 2013, **22**(7):517-521.
23. Witt O, Deubzer HE, Milde T, Oehme I: **HDAC family: What are the cancer relevant targets?** *Cancer Lett* 2009, **277**(1):8-21.

CHAPTER 7

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

A group of severe autoimmune disorders, comprising granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA), is characterized by inflammation that predominantly affects the small- to medium-sized blood vessels [1]. A hallmark feature of these diseases is the presence of circulating anti-neutrophil cytoplasmic antibodies (ANCA). ANCA are primarily directed against proteinase 3 (PR3) or myeloperoxidase (MPO) that are enzymes contained in the azurophilic granules of neutrophils and monocytes [1, 2]. As discussed in **chapters 1** and **2**, there is substantial evidence indicating that ANCA are pathogenic and that ANCA-mediated vascular damage is initiated by a pre-existing inflammatory environment [3]. In the presence of pro-inflammatory stimuli, neutrophils and monocytes are pre-activated (primed), which leads to PR3 and MPO translocation to the cell surface where the antigens are accessible to the autoantibodies [4]. ANCA binding to their antigens fully activates neutrophils and monocytes via Fc receptor mediated interactions [5], resulting in production of pro-inflammatory cytokines, neutrophil degranulation and release of reactive oxygen species (ROS) [6, 7] which damage the endothelium and adjacent tissues. Although it is still not known what triggers the onset of ANCA-associated vasculitides (AAV), it is clear that these are multifactorial conditions in which environmental factors [8], genetic predisposition [9] and disturbances in immune regulation [10] contribute to disease development and progression.

The recent clinical observations that AAV can be effectively treated with B cell depletion therapy highlight the important role of B cells in the pathogenesis of these diseases [11, 12]. Under physiological circumstances, B cells regulate immune responses through generation of antibodies, production of pro- and anti-inflammatory cytokines, antigen presentation and T cell co-stimulation [13]. It is now recognized that in AAV B cells contribute to the disease process through autoantibody-dependent as well as autoantibody-independent mechanisms. ANCA-mediated pathogenic effects have been widely studied *in vitro* and *in vivo* [5, 6, 14, 15]. Although it is still not known what factors mediate ANCA production in the first place, the available evidence suggests that the generation of ANCA-specific B cells is a T cell-dependent process [16, 17]. Nevertheless, B cells and their antibody-independent functions have not been extensively studied in AAV. In this thesis we investigated the mechanisms that are involved in B cell activation and autoantibody production in AAV patients. Besides antibody-dependent functions, we also studied B cell antibody-independent functions. Hence, we examined the capacity of B cells to suppress activation of other immune cells in patients with AAV and studied mechanisms that regulate cytokine production in primary human B cells.

An overview of the immunological mechanisms that potentially contribute to the pathogenesis of AAV is given in **chapter 2**. In particular, we have discussed the evidence that CD4+ T helper (Th) cell subsets are critically involved in many aspects of AAV pathogenesis, including the generation of PR3- and MPO-specific autoreactive B cells and the effector mechanisms causing vascular damage and granuloma formation [10].

In addition to the well-described Th1, Th2, Th17 and regulatory T cell (T_{REG}) subsets [18], a Th cell population characterized by the production of IL-21 has been described

more recently [19]. IL-21-producing Th cells provide help to B cells by stimulating B cell humoral responses [20]. Because in AAV autoantibodies are considered key mediators in the pathogenic process that leads to vascular damage [2], we studied the frequencies of IL-21-producing CD4+ Th cells in patients with GPA in **chapter 3**. These studies revealed that in GPA patients the proportion of IL-21+ Th cells is increased when compared to age- and gender-matched healthy individuals. Furthermore, GPA patients that were ANCA sero-negative at the time of inclusion had a lower percentage of IL-21-producing Th cells than patients which were ANCA seropositive. As IL-21 together with B cell activating factor (BAFF) promotes B cell class-switching and differentiation towards plasma cells [21, 22], we hypothesized that the increased percentage of IL-21-producing Th cells may be an important contributing factor in the production of ANCA. To test this hypothesis, we evaluated the effect of IL-21 and BAFF on autoantibody production *in vitro* and found that the combined treatment of IL-21 and BAFF stimulated PR3-ANCA production. This phenomenon was studied further in **chapter 4**, where we demonstrated that both endogenous and exogenous factors contribute to autoantibody production. PR3-ANCA production stimulated by IL-21 and BAFF could be further enhanced by the addition of the Toll-like receptor 9 (TLR9) agonist CpG-ODN. Moreover, we found that treatment with CpG-ODN up-regulated IL-21 receptor (IL-21R) expression on B cells. This could explain why the combined treatment of CpG-ODN, BAFF and IL-21 was a more potent inducer of (auto)antibody production than BAFF and IL-21 alone as up-regulation of IL-21R might sensitize B cells to become more responsive to IL-21 stimulation. Interestingly, GPA patients had increased percentages of circulating IL-21R+ memory B cells when compared to HC, which suggests that also *in vivo* memory B cells in GPA patients might be more sensitive for stimulation through the IL-21R.

Recently, it was shown that peripheral blood mononuclear cells (PBMC) from rheumatoid arthritis (RA) patients can be stimulated to produce anti-citrullinated protein antibodies (ACPA) *in vitro* upon stimulation with anti-IgM, IL-21, BAFF and CD40 ligand (CD40L) [23]. In this study, it was demonstrated that ACPA production *in vitro* strongly correlated with the ACPA serum titer. Also, in some PBMC cultures from RA patients spontaneous ACPA production was found. Spontaneously produced ACPA could not be eliminated by depletion of CD20+ cells from the PBMC culture, indicating that ACPA is produced by circulating CD20- plasmablasts or plasma cells [23]. In **chapter 4** we also observed that *in vitro* ANCA production strongly correlated with the serum ANCA titer, suggesting ongoing and persistent generation of autoreactive B cells which enter the circulation. Moreover, spontaneous ANCA production was observed in PBMC cultures of some GPA patients and it would be of particular interest to study whether depletion of CD20+ B cells can abolish spontaneous ANCA production. In the rituximab trial reported by Stone and colleagues, approximately 50% of AAV patients that were treated with B cell depletion therapy became ANCA-negative at 6 months after the start of the therapy [11]. In the remaining 50% of patients, ANCA were not eliminated by depletion of CD20+ cells, suggesting that in some patients ANCA is either produced by CD20- cells or ANCA-producing cells reside in tissues and are

shielded from the effects of rituximab which is administered systemically. Especially in these cases where ANCA production cannot be eliminated by depleting CD20+ cells, it will be of interest to test whether treatment with anti-BAFF or anti-IL-21 monoclonal antibodies, can diminish autoantibody production as both BAFF and IL-21 have been proposed as potential therapeutic targets for AAV patients [24, 25]. In addition to its effects on B cells, IL-21 is known to promote the generation of Th17 cells [26], which is also supported by the findings of our study as we found a positive correlation between the frequencies of IL-21+ Th cells and IL-17+ Th cells (**chapter 3**). As demonstrated by us and others, the proportion of Th17 cells is increased in AAV patients and these cells are considered essential players in the inflammatory process [27-29]. An important question therefore is whether inhibition of IL-21 or blockade of the IL-21R would also diminish the proportion of Th17 cells in AAV patients. Taken together, inhibition of IL-21 may be an attractive therapeutic target in patients with AAV and autoimmune diseases in general due to its dual inhibitory effect on humoral and cellular immune responses but further studies are clearly needed to establish this more firmly.

Until recently, B cells were generally perceived as effector cells due to their ability to produce (auto)antibodies. As opposed to this concept, a subset of B cells termed regulatory B cells (B_{REG}) has been reported to have an immune suppressive function in humans [13, 30]. The B_{REG} subset has gained considerable interest in recent years as a potential modulator of immune responses [31, 32] in particular when it was demonstrated that B_{REGS} are functionally defective in some autoimmune diseases, including SLE [31] and RA [33]. Because B cells or their regulatory function have not been extensively studied in AAV patients, we analyzed the distribution of the circulating B cell subsets in patients with AAV and age- and gender-matched healthy controls in **chapter 5**. Besides the transitional, naive and memory B cell subsets, we also characterized the frequencies of CD19+CD24^{high}CD38^{high} and CD19+CD24^{high}CD27+ B cells since these markers have been proposed to phenotypically distinguish B_{REGS} in humans [31, 32]. Overall, we observed dysregulated homeostasis of the circulating B cell subsets in AAV patients. CD19+CD24^{high}CD38^{high} cells were decreased in AAV patients during active disease, whereas the percentage of CD19+CD24^{high}CD27+ cells was diminished independent of disease activity. As IL-10 is considered the functional marker for B_{REGS}, we also analyzed the percentage of IL-10-producing B cells in AAV patients and HC. However, the percentage of IL-10+ B cells did not differ between patients and HC. Finally, in order to directly assess the suppressive function of B cells, we studied the capacity of B cells to inhibit TNF-α production by monocytes upon LPS stimulation. B cells from AAV patients in remission suppressed monocyte activation to a similar extent as B cells from HC, indicating that the suppressive function of B cells is not disturbed in AAV patients during clinical remission. In this study, the suppressive function of B_{REGS} was not investigated in patients with active disease; therefore, we cannot exclude the possibility that at the time of active disease, the inhibitory capacity of B_{REGS} is impaired. An intriguing question to be addressed in future studies is whether a steady and robust B_{REG} compartment is critical for maintaining stable remission in AAV patients.

Immune-suppressive effects of B cells have also been studied in the setting of transplantation. Increased frequencies of transitional and naïve B cells in peripheral blood are associated with renal transplant tolerance in humans [34]. In addition, a study by Nouël *et al*. showed that the B cell suppressive function is impaired in kidney transplantation patients with chronic-antibody mediated rejection [35]. As cellular therapies based on *ex vivo* induction of T_{REG} cells are being tested in clinical trials as an immunosuppressive treatment for transplantation patients [36], there is growing interest in the potential use of B_{REGS} for cellular therapy in transplantation and autoimmunity [37]. However, before such studies can be performed, several major questions with respect to B_{REG} phenotypes and functions need to be addressed. B cells producing IL-10 can be found in the transitional, naive and memory B cell compartments, suggesting that B cells can acquire a suppressive function during different stages of maturation [31, 38]. Additionally, a study by Maseda *et al*. demonstrated that the ability of B cells to produce IL-10 is transient and occurs before differentiating into antibody-producing cells [39], suggesting that B_{REGS} are functionally highly plastic cell population rather than a stable subset. For this reason the use of B_{REGS} for cellular therapy might be particularly challenging. Identification of a specific transcription factor that regulates IL-10 production in B cells would increase our understanding on how B_{REGS} are induced and sustained, which is crucial for their potential clinical applications. Although stimulation through TLR9 is a widely used method for induction of IL-10 production in human B cells [31, 32, 38], it is unclear why only a subset of B cells becomes competent to produce IL-10 when all B cells express TLR9 [40]. One explanation may be that additional layers of regulation, e.g., epigenetic modifications or microRNAs, are involved in controlling IL-10 production in B cells.

Work with CD4+ Th cells has demonstrated that commitment to a certain Th cell lineage and production of lineage-specific cytokines is epigenetically regulated [41]. For example, conversion from T_{REG} to Th17 cells is regulated by histone deacetylases (HDAC) and inhibition of HDACs suppresses Th17 cell formation from T_{REG} cells [42]. Thus, in **chapter 6**, we questioned if the regulation of pro- and anti-inflammatory cytokine production in primary human B cells is dependent on histone/protein acetylases. To this end, we tested the effect of the HDAC inhibitor trichostatin A (TSA) on the production of anti- and pro-inflammatory cytokines in primary human B cells. We found that TSA had an inhibitory effect on IL-10, IL-6 and TGF-β1 expression, but, at the same time, it promoted production of TNF-α in B cells. In addition, TSA increased the acetylation status of histone 3 (H3) and histone 4 (H4) in the promoter regions of *IL-10*, *TNF*-α, *IL-6* and *TGF*-β*1* genes. Because acetylation of H3 and H4 are permissive epigenetic marks associated with active gene transcription [41], the alterations in histone acetylation could not explain the observed effect on IL-10 and IL-6 expression. Because histone acetylases regulate the acetylation status not only of histones but also of non-histone proteins, it is likely that TSA affects the signaling pathways that mediate the production of these cytokines. As TNF-α and IL-6 have been shown to be NF-κB-responsive genes [43], we further investigated whether TSA

mediates its effects through NF-κB. Inhibition of IKK-β, which is required for activation of the classical NF-κB pathway [44], further enhanced TSA-induced IL-10 and IL-6 inhibition. On the other hand, the inhibitor Bay 11-7082, which non-specifically inhibits the activation of both classical and non-classical NF-κB pathways, partially blocked TSA-induced TNF-α production. Overall, these data demonstrate that HDACs regulate cytokine production in human B cells although the exact signaling pathways that are affected by TSA are yet to be identified.

HDAC inhibitors are considered promising therapeutic agents for cancer treatment and one broad-spectrum HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) has been approved for treatment of advanced cutaneous T cell lymphoma (CTCL) [45, 46]. However, due to adverse effects there is interest in developing isoform-specific HDAC inhibitors with a wider therapeutic window [46]. Reduction of undesirable side effects of these drugs probably would also permit their clinical testing for other indications, such as autoimmunity and transplant rejection. In the study described in **chapter 6**, we used TSA which non-specifically inhibits class I and II HDACs. Future studies should investigate which HDAC isoforms are primarily involved in the production of certain cytokines in B cells. Such studies might provide further opportunities to down-regulate or up-regulate B cell cytokine production in a targeted way. In the light of recent work that proposed that B cell depletion therapy is beneficial due to elimination of IL-6-producing B cells [47], targeting B cells with the aim to modulate their cytokine expression profile might be a novel approach for immunomodulation therapy.

In recent years, significant progress has been made towards a better understanding of AAV disease pathogenesis. A genome-wide association study (GWAS) in AAV demonstrated that patients with GPA and MPA have different genetic backgrounds which were associated with ANCA specificity [9]. These data indicate that PR3-AAV and MPO-AAV are two distinct syndromes rather than two sub-types of the same disease. Particularly for research studies, which investigate intricate molecular and cellular mechanisms, these patient groups should be studied separately as PR3-ANCA and MPO-ANCA disease might have a different underlying pathogenesis.

A longstanding question in the ANCA field is whether ANCA are pathogenic or not. *In vitro* studies have convincingly demonstrated pathogenic consequences of ANCA binding to its target antigens [6, 7] and ANCA is well-established and universally used as a specific marker for the diagnosis of small vessel vasculitides. Yet, the prognostic value of ANCA for predicting relapses and monitoring disease activity is controversial as ANCA titers correlate with disease activity in some but certainly not all patients [48]. A recent study by Roth *et al.* demonstrated that in MPO-ANCA patients during active disease MPO-ANCA is directed against different epitope of MPO than during quiescent disease [49], suggesting that epitope-specificity is crucial for pathogenic or non-pathogenic effects of these autoantibodies. Currently, diagnostic tests only screen for the presence of ANCA and epitope specificity is not taken into consideration. However, developing an epitope-specific ANCA assay which would allow distinguishing between ANCA directed against non-pathogenic and pathogenic

epitopes could be of clinical relevance and possibly of prognostic value for disease activity. It also might explain the existing controversy regarding ANCA pathogenicity.

Clinically, the most influential advance in recent years has been the demonstration that B cell depletion therapy is an efficacious approach for the treatment of AAV [11, 12, 50, 51]. Interestingly, as patients were observed to achieve clinical remission before the clearance of ANCA from the circulation, it suggests that B cells substantially contribute to ANCA disease pathogenesis via autoantibody-independent mechanisms [11]. Moreover, despite the elimination of B_{REGS}, AAV patients benefit from B cell depletion therapy, suggesting that the pathogenic effect of B cells overrules their regulatory and suppressive potential. As indicated by previous studies, B cell depletion might have a substantial effect on the T cell compartment, including a reduction of Th17 cells and improved function of T_{REGS} [52-54]. Future studies should determine whether beneficial alterations in the T cell compartment also occur in AAV patients upon B cell depletion therapy. Furthermore, it was demonstrated recently that B cells are involved in monocyte migration through production of the chemokine CCL7, which activates monocyte migration from the bone marrow [55]. Whether B cells also contribute to immune cell recruitment and migration to the site of inflammation in patients with inflammatory and autoimmune diseases is an intriguing concept to be investigated in future studies.

In conclusion, the data presented in this thesis contribute to the knowledge on the humoral and cellular functions of B cells in AAV. We have shown that BAFF and IL-21 augment ANCA production *in vitro*, an effect that could be further enhanced by activation of TLR9. Although in AAV patients the homeostasis of circulating B cells is disturbed, their ability to produce IL-10 and limit monocyte activation is not impaired. This work demonstrates that characterization of the B cell compartment based solely on phenotypic markers does not provide conclusive data on their functionality. Future studies should be performed to more directly assess the function of B cells, e.g., via more extensive profiling of B cell cytokine production at different stages of disease activity. Furthermore, we have shown that cytokine production in primary human B cells is regulated by HDACs but additional studies are needed to reveal if targeted interference with HDAC activity is an attractive approach to modulate pro- and anti-inflammatory cytokine production in B cells. Overall, a more detailed understanding of B cell properties in AAV pathogenesis will aid in improved disease monitoring and may provide clues to more specific drug targets for treatment.

REFERENCES

1. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, Flores-Suarez LF, Gross WL, Guillevin L, Hagen EC, Hoffman GS, Jayne DR, Kallenberg CG, Lamprecht P, Langford CA, Luqmani RA, Mahr AD, Matteson EL, Merkel PA, Ozen S, Pusey CD, Rasmussen N, Rees AJ, Scott DG, Specks U, Stone JH, Takahashi K, Watts RA: **2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides.** *Arthritis Rheum* 2013, **65**(1):1-11.
2. Jennette JC, Falk RJ, Hu P, Xiao H: **Pathogenesis of antineutrophil cytoplasmic autoantibody-associated small-vessel vasculitis.** *Annu Rev Pathol* 2013, **8**:139-160.
3. Kallenberg CG, Stegeman CA, Abdulahad WH, Heeringa P: **Pathogenesis of ANCA-associated vasculitis: new possibilities for intervention.** *Am J Kidney Dis* 2013, **62**(6):1176-1187.
4. Hu N, Westra J, Kallenberg CG: **Dysregulated neutrophil--endothelial interaction in antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides: implications for pathogenesis and disease intervention.** *Autoimmun Rev* 2011, **10**(9):536-543.
5. Mulder AH, Heeringa P, Brouwer E, Limburg PC, Kallenberg CG: **Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA): a Fc gamma RII-dependent process.** *Clin Exp Immunol* 1994, **98**(2):270-278.
6. Falk RJ, Terrell RS, Charles LA, Jennette JC: **Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro.** *Proc Natl Acad Sci U S A* 1990, **87**(11):4115-4119.
7. Kettritz R, Jennette JC, Falk RJ: **Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils.** *J Am Soc Nephrol* 1997, **8**(3):386-394.
8. Chen M, Kallenberg CG: **The environment, geoepidemiology and ANCA-associated vasculitides.** *Autoimmun Rev* 2010, **9**(5):A293-8.
9. Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, Baslund B, Brenchley P, Bruchfeld A, Chaudhry AN, Cohen Tervaert JW, Deloukas P, Feighery C, Gross WL, Guillevin L, Gunnarsson I, Harper L, Hruskova Z, Little MA, Martorana D, Neumann T, Ohlsson S, Padmanabhan S, Pusey CD, Salama AD, Sanders JS, Savage CO, Segelmark M, Stegeman CA, Tesar V, Vaglio A, Wieczorek S, Wilde B, Zwerina J, Rees AJ, Clayton DG, Smith KG: **Genetically distinct subsets within ANCA-Associated Vasculitis.** *N Engl J Med* 2012, **367**(3):214-223.
10. Lepse N, Abdulahad WH, Kallenberg CG, Heeringa P: **Immune regulatory mechanisms in ANCA-associated vasculitides.** *Autoimmun Rev* 2011, **11**(2):77-83.
11. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, Kallenberg CG, St Clair EW, Turkiewicz A, Tchao NK, Webber L, Ding L, Sejismundo LP, Mieras K, Weitzenkamp D, Ikle D, Seyfert-Margolis V, Mueller M, Brunetta P, Allen NB, Fervenza FC, Geetha D, Keogh KA, Kissin EY, Monach PA, Peikert T, Stegeman C, Ytterberg SR, Specks U, RAVE-ITN Research Group: **Rituximab versus cyclophosphamide for ANCA-associated vasculitis.** *N Engl J Med* 2010, **363**(3):221-232.
12. Jones RB, Tervaert JW, Hauser T, Luqmani R, Morgan MD, Peh CA, Savage CO, Segelmark M, Tesar V, van Paassen P, Walsh D, Walsh M, Westman K, Jayne DR, European Vasculitis Study Group: **Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis.** *N Engl J Med* 2010, **363**(3):211-220.
13. Lund FE, Randall TD: **Effector and regulatory B cells: modulators of CD4(+) T cell immunity.** *Nat Rev Immunol* 2010, **10**(4):236-247.
14. Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, Maeda N, Falk RJ, Jennette JC: **Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice.** *J Clin Invest* 2002, **110**(7):955-963.
15. Little MA, Al-Ani B, Ren S, Al-Nuaimi H, Leite M,Jr, Alpers CE, Savage CO, Duffield JS: **Anti-proteinase 3 anti-neutrophil cytoplasm autoantibodies recapitulate systemic vasculitis in mice with a humanized immune system.** *PLoS One* 2012, **7**(1):e28626.
16. Brouwer E, Tervaert JW, Horst G, Huitema MG, van der Giessen M, Limburg PC, Kallenberg CG: **Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis and clinically related disorders.** *Clin Exp Immunol* 1991, **83**(3):379-386.
17. Brouwer E, Stegeman CA, Huitema MG, Limburg PC, Kallenberg CG: **T cell reactivity to proteinase 3 and myeloperoxidase in**

patients with Wegener's granulomatosis (WG). *Clin Exp Immunol* 1994, **98**(3):448-453.

18. Pepper M, Jenkins MK: **Origins of CD4(+) effector and central memory T cells.** *Nat Immunol* 2011, **12**(6):467-471.
19. Crotty S: **Follicular helper CD4 T cells (TFH).** *Annu Rev Immunol* 2011, **29**:621-663.
20. Tangye SG, Ma CS, Brink R, Deenick EK: **The good, the bad and the ugly - TFH cells in human health and disease.** *Nat Rev Immunol* 2013, **13**(6):412-426.
21. Ettinger R, Sims GP, Robbins R, Withers D, Fischer RT, Grammer AC, Kuchen S, Lipsky PE: **IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells.** *J Immunol* 2007, **178**(5):2872-2882.
22. Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, Leonard WJ, Lipsky PE: **IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells.** *J Immunol* 2005, **175**(12):7867-7879.
23. Kerkman PF, Rombouts Y, van der Voort EI, Trouw LA, Huizinga TW, Toes RE, Scherer HU: **Circulating plasmablasts/plasmacells as a source of anticitrullinated protein antibodies in patients with rheumatoid arthritis.** *Ann Rheum Dis* 2013, **72**(7):1259-1263.
24. [http://clinicaltrials.gov/show/NCT01663623]
25. Abdulahad WH, Lepse N, Stegeman CA, Huitema MG, Doornbos-van der Meer B, Tadema HH, Rutgers A, Limburg PC, Kallenberg CG, Heeringa P: **Increased frequency of circulating IL-21 producing Th-cells in patients with granulomatosis with polyangiitis.** *Arthritis Res Ther* 2013, **15**(3):R70.
26. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK: **IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells.** *Nature* 2007, **448**(7152):484-487.
27. Abdulahad WH, Stegeman CA, Limburg PC, Kallenberg CG: **Skewed distribution of Th17 lymphocytes in patients with Wegener's granulomatosis in remission.** *Arthritis Rheum* 2008, **58**(7):2196-2205.
28. Nogueira E, Hamour S, Sawant D, Henderson S, Mansfield N, Chavele KM, Pusey CD, Salama AD: **Serum IL-17 and IL-23 levels and autoantigen-specific Th17 cells are elevated in patients with ANCA-associated vasculitis.** *Nephrol Dial Transplant* 2010, **25**(7):2209-2217.
29. Wilde B, Thewissen M, Damoiseaux J, Hilhorst M, van Paassen P, Witzke O, Cohen Tervaert JW: **Th17 expansion in granulomatosis with polyangiitis (Wegener's): the role of disease activity, immune regulation and therapy.** *Arthritis Res Ther* 2012, **14**(5):R227.
30. Mauri C: **Regulation of immunity and autoimmunity by B cells.** *Curr Opin Immunol* 2010, **22**(6):761-767.
31. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, Mauri C: **CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients.** *Immunity* 2010, **32**(1):129-140.
32. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, Szabolcs PM, Bernstein SH, Magro CM, Williams AD, Hall RP, St Clair EW, Tedder TF: **Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells.** *Blood* 2011, **117**(2):530-541.
33. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, Mauri C: **CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation.** *Sci Transl Med* 2013, **5**(173):173ra23.
34. Newell KA, Asare A, Kirk AD, Gisler TD, Bourcier K, Suthanthiran M, Burlingham WJ, Marks WH, Sanz I, Lechler RI, Hernandez-Fuentes MP, Turka LA, Seyfert-Margolis VL, Immune Tolerance Network ST507 Study Group: **Identification of a B cell signature associated with renal transplant tolerance in humans.** *J Clin Invest* 2010, **120**(6):1836-1847.
35. Nouel A, Segalen I, Jamin C, Doucet L, Caillard S, Renaudineau Y, Pers JO, Le Meur Y, Hillion S: **B cells display an abnormal distribution and an impaired suppressive function in patients with chronic antibody-mediated rejection.** *Kidney Int* 2013, :.
36. Safinia N, Leech J, Hernandez-Fuentes M, Lechler R, Lombardi G: **Promoting transplantation tolerance; adoptive regulatory T cell therapy.** *Clin Exp Immunol* 2013, **172**(2):158-168.
37. Nouel A, Simon Q, Jamin C, Pers JO, Hillion S: **Regulatory B Cells: An Exciting Target for Future Therapeutics in Transplantation.** *Front Immunol* 2014, **5**:11.
38. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG, Ruckert

B, Akdis CA, Akdis M: **IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses.** *J Allergy Clin Immunol* 2013, **131**(4):1204-1212.

39. Maseda D, Smith SH, DiLillo DJ, Bryant JM, Candando KM, Weaver CT, Tedder TF: **Regulatory B10 cells differentiate into antibody-secreting cells after transient IL-10 production in vivo.** *J Immunol* 2012, **188**(3):1036-1048.
40. Tadema H, Abdulahad WH, Stegeman CA, Kallenberg CG, Heeringa P: **Increased expression of Toll-like receptors by monocytes and natural killer cells in ANCA-associated vasculitis.** *PLoS One* 2011, **6**(9):e24315.
41. Wilson CB, Rowell E, Sekimata M: **Epigenetic control of T-helper-cell differentiation.** *Nat Rev Immunol* 2009, **9**(2):91-105.
42. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I: **Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells.** *Blood* 2008, **112**(6):2340-2352.
43. Ahn KS, Aggarwal BB: **Transcription factor NF-kappaB: a sensor for smoke and stress signals.** *Ann N Y Acad Sci* 2005, **1056**:218-233.
44. Liu F, Xia Y, Parker AS, Verma IM: **IKK biology.** *Immunol Rev* 2012, **246**(1):239-253.
45. Duvic M, Vu J: **Vorinostat: a new oral histone deacetylase inhibitor approved for cutaneous T-cell lymphoma.** *Expert Opin Investig Drugs* 2007, **16**(7):1111-1120.
46. Balasubramanian S, Verner E, Buggy JJ: **Isoform-specific histone deacetylase inhibitors: the next step?** *Cancer Lett* 2009, **280**(2):211-221.
47. Barr TA, Shen P, Brown S, Lampropoulou V, Roch T, Lawrie S, Fan B, O'Connor RA, Anderton SM, Bar-Or A, Fillatreau S, Gray D: **B cell depletion therapy ameliorates autoimmune disease through ablation of IL-6-producing B cells.** *J Exp Med* 2012, **209**(5):1001-1010.
48. Kerr GS, Fleisher TA, Hallahan CW, Leavitt RY, Fauci AS, Hoffman GS: **Limited prognostic value of changes in antineutrophil cytoplasmic antibody titer in patients with Wegener's granulomatosis.** *Arthritis Rheum* 1993, **36**(3):365-371.
49. Roth AJ, Ooi JD, Hess JJ, van Timmeren MM, Berg EA, Poulton CE, McGregor J, Burkart M, Hogan SL, Hu Y, Winnik W, Nachman PH, Stegeman CA, Niles J, Heeringa P, Kitching AR, Holdsworth S, Jennette JC, Preston GA, Falk RJ: **Epitope specificity determines pathogenicity and detectability in ANCA-associated vasculitis.** *J Clin Invest* 2013, **123**(4):1773-1783.
50. Smith RM, Jones RB, Guerry MJ, Laurino S, Catapano F, Chaudhry A, Smith KG, Jayne DR: **Rituximab for remission maintenance in relapsing antineutrophil cytoplasmic antibody-associated vasculitis.** *Arthritis Rheum* 2012, **64**(11):3760-3769.
51. Specks U, Merkel PA, Seo P, Spiera R, Langford CA, Hoffman GS, Kallenberg CG, St Clair EW, Fessler BJ, Ding L, Viviano L, Tchao NK, Phippard DJ, Asare AL, Lim N, Ikle D, Jepson B, Brunetta P, Allen NB, Fervenza FC, Geetha D, Keogh K, Kissin EY, Monach PA, Peikert T, Stegeman C, Ytterberg SR, Mueller M, Sejismundo LP, Mieras K, Stone JH, RAVE-ITN Research Group: **Efficacy of remission-induction regimens for ANCA-associated vasculitis.** *N Engl J Med* 2013, **369**(5):417-427.
52. Ait-Oufella H, Herbin O, Bouaziz JD, Binder CJ, Uyttenhove C, Laurans L, Taleb S, Van Vre E, Esposito B, Vilar J, Sirvent J, Van Snick J, Tedgui A, Tedder TF, Mallat Z: **B cell depletion reduces the development of atherosclerosis in mice.** *J Exp Med* 2010, **207**(8):1579-1587.
53. van de Veerdonk FL, Lauwerys B, Marijnissen RJ, Timmermans K, Di Padova F, Koenders MI, Gutierrez-Roelens I, Durez P, Netea MG, van der Meer JW, van den Berg WB, Joosten LA: **The anti-CD20 antibody rituximab reduces the Th17 cell response.** *Arthritis Rheum* 2011, **63**(6):1507-1516.
54. Hamel KM, Cao Y, Ashaye S, Wang Y, Dunn R, Kehry MR, Glant TT, Finnegan A: **B cell depletion enhances T regulatory cell activity essential in the suppression of arthritis.** *J Immunol* 2011, **187**(9):4900-4906.
55. Zouggari Y, Ait-Oufella H, Bonnin P, Simon T, Sage AP, Guerin C, Vilar J, Caligiuri G, Tsiantoulas D, Laurans L, Dumeau E, Kotti S, Bruneval P, Charo IF, Binder CJ, Danchin N, Tedgui A, Tedder TF, Silvestre JS, Mallat Z: **B lymphocytes trigger monocyte mobilization and impair heart function after acute myocardial infarction.** *Nat Med* 2013, **19**(10):1273-1280.

CHAPTER 8

NEDERLANDSE SAMENVATTING

ACHTERGROND

Vasculitis is een ziekteproces dat gekenmerkt wordt door ontsteking van bloedvaten [1]. De vaatontsteking kan de bloeddoorstroming belemmeren, waardoor de toevoer van zuurstof en voedingstoffen naar de organen wordt verstoord. In ernstige gevallen kan dit leiden tot falen van het aangedane orgaan. In principe kunnen alle soorten bloedvaten in ieder orgaan ontstoken raken. Vasculitiden worden onderverdeeld in twee groepen: primaire vasculitiden en secundaire vasculitiden. Bij primaire vasculitiden staat de ontsteking van de bloedvaten op de voorgrond en is de oorzaak van de aandoening grotendeels onbekend. Bij secundaire vasculitiden is er een onderliggende oorzaak aan te wijzen voor de vasculitis, zoals een infectie [1]. Daarnaast worden vasculitiden onderverdeeld op basis van de grootte van de aangedane bloedvaten en karakteristieke afwijkingen bij microscopisch weefselonderzoek [1].

Een groep van vasculitiden waartoe granulomatose met polyangiitis (GPA), microscopische polyangiitis (MPA) en eosinofiele granulomatose met polyangiitis (EGPA) behoren, wordt gekenmerkt door ontstekingen van voornamelijk de kleine bloedvaten, zoals capillairen en postcapillaire venules [1, 2]. Deze aandoeningen kunnen leiden tot ziektemanifestaties in organen zoals de huid, longen en nieren. Kenmerkend voor deze vormen van kleine vaten vasculitis is dat ze sterk geassocieerd zijn met de aanwezigheid van autoantistoffen gericht tegen eiwitten in de korrels van neutrofiele granulocyten en monocyten [1]. Deze autoantistoffen, ook wel anti-neutrofiele cytoplasmatische autoantistoffen genoemd (ANCA), zijn met name gericht tegen proteinase 3 (PR3) en myeloperoxidase (MPO) [2].

De sterke associatie van ANCA met het optreden van kleine vaten vasculitiden doet vermoeden dat deze autoantistoffen een belangrijke rol spelen in het ontstaan van deze ziekten. In de afgelopen 25 jaar zijn in experimentele en klinische studies inderdaad aanwijzingen gevonden dat deze autoantistoffen een belangrijke rol spelen in het ziekteproces, met name wanneer er al een lichte vorm van ontsteking aanwezig is [3]. Experimentele studies met geïsoleerde neutrofiele granulocyten en monocyten hebben aangetoond dat deze cellen de ANCA-antigenen PR3 en MPO tot expressie kunnen brengen op hun celmembraan na stimulatie met pro-inflammatoire cytokines [4]. Wanneer ANCA binden aan membraangebonden PR3 en MPO, veroorzaakt dit verdere productie van pro-inflammatoire cytokines door de neutrofiele granulocyten en monocyten, evenals degranulatie en productie van zuurstofradicalen [5-7]. Deze stoffen kunnen vervolgens vaat -en weefselschade veroorzaken. Tot nu toe is onbekend waarom dit ontstekingsproces optreedt in patiënten met ANCA-geassocieerde vasculitiden. Het meest waarschijnlijk is dat deze aandoeningen optreden door een combinatie van omgevingsfactoren [8], genetische factoren [9] en een verstoorde regulatie van immuunreacties [10].

Recente klinische studies hebben aangetoond dat ANCA-geassocieerde vasculitiden effectief behandeld kunnen worden middels therapeutische depletie van circulerende B-cellen. Dit suggereert dat B-cellen een prominente rol spelen in het ziekteproces [11, 12]. B-cellen dragen normaliter bij aan immuniteit tegen ziekteverwekkers door productie van antistoffen. Daarnaast kunnen B-cellen ook

immuunmodulerende cytokines produceren en antigenen presenteren aan T-cellen [13]. Deze antistofafhankelijke en -onafhankelijke processen spelen waarschijnlijk ook een belangrijke rol in het ontstaan van kleine vaten vasculitiden [5, 6, 14-17]. Hoewel er al veel bekend is over de antistofafhankelijke bijdrage van B-cellen aan het ontstaan van de ANCA-geassocieerde vasculitiden, is er weinig bekend over de antistofonafhankelijke werking van B-cellen in deze aandoeningen.

DIT PROEFSCHRIFT

Het onderzoek zoals beschreven in dit proefschrift heeft zich met name gericht op de mechanismen die een rol zouden kunnen spelen bij de ontwikkeling van autoantistoffen in ANCA-geassocieerde vasculitiden. Daarnaast is ook de antistofonafhankelijke werking van B-cellen in het ziekteproces bestudeerd en zijn de regulerende mechanismen onderzocht die de productie van cytokines door humane B-cellen beïnvloeden.

In **hoofdstuk 2** wordt een overzicht gegeven van de huidige kennis over de immunologische mechanismen die bijdragen aan het ontstaan van ANCA-geassocieerde kleine vaten vasculitiden. In dit overzicht wordt met name ingegaan op de cruciale rol van CD4+ T helper (Th)-cellen in de inductie van PR3- en MPO-specifieke autoreactieve B-cellen en hun bijdrage aan de effector fase van het ziekteproces dat uiteindelijk leidt tot schade aan de vaatwand en weefsels [10].

Binnen de Th-populatie kunnen verschillende subpopulaties worden onderscheiden die elk een unieke functie hebben in de immuunrespons. Een recent beschreven populatie van Th-cellen wordt gekarakteriseerd door de productie van IL-21, een cytokine die met name een rol speelt bij het stimuleren van antilichaamproductie door B-cellen [18-20]. Aangezien autoantilichamen van belang zijn in ANCA-geassocieerde kleine vaten vasculitiden, werd in **hoofdstuk 3** de frequentie van IL-21-producerende CD4+ Th-cellen bepaald in GPA-patiënten. Deze studies lieten zijn dat in GPA-patiënten het percentage IL-21-producerende Th-cellen is toegenomen in vergelijking met gezonde personen. Ook werd aangetoond dat patiënten die op het moment van inclusie ANCA-negatief waren, een lager percentage van IL-21-producerende Th-cellen hadden dan patiënten die ANCA-positief waren. Vanuit de literatuur is bekend dat IL-21 tezamen met de B-cell activating Factor (BAFF) zowel de switch in antistofsubklasse als de differentiatie van B-cellen naar plasmacellen bevordert [21, 22]. De hypothese was dan ook dat een toename van IL-21-producerende Th-cellen bijdraagt aan de productie van ANCA. Deze hypothese werd getoetst in experimentele studies waarin werd aangetoond dat incubatie van perifere bloed mononucleaire cellen (PBMC) van GPA-patiënten met IL-21 en BAFF inderdaad leidde tot PR3-ANCA productie. Dit fenomeen werd verder bestudeerd in **hoofdstuk 4** waarin werd gedemonstreerd dat een combinatie van endogene en exogene factoren bijdragen aan de productie van autoantistoffen. *In vitro* productie van IL-21/BAFF-geïnduceerde PR3-ANCA door PBMC kon verder worden gestimuleerd door toevoeging van een agonist van Toll-like receptor (TLR) 9, CpG-oligodeoxynucleotiden (CpG-ODN). CpG–ODN is vergelijkbaar met ongemethyleerde

vormen van DNA die met name in bacteriën voorkomen en de immuunrespons kunnen stimuleren. Verder werd aangetoond dat behandeling met CpG-ODN het aantal IL-21-receptoren op B-cellen opreguleert. Deze observatie zou kunnen verklaren waarom de combinatie van CpG-ODN, BAFF en IL-21 meer antilichaamproductie induceert dan BAFF en IL-21 alleen. De CpG-gemedieerde toename van IL-21-receptoren op B-cellen maakt deze cellen gevoeliger voor de effecten van IL-21. Een interessante bevinding was dat in de circulatie van GPA-patiënten inderdaad een toegenomen percentage van IL-21-producerende geheugen T-cellen aangetoond kon worden. Deze data suggereren dat blokkade van de IL-21-receptor van therapeutische waarde zou kunnen zijn in patiënten met ANCA-geassocieerde kleine vaten vasculitis.

Tot voor kort werden B-cellen voornamelijk beschouwd als cellen die antistoffen produceren. Recente onderzoeken hebben echter aangetoond dat specifieke B-celsubsets bestaan die van belang zijn voor de onderdrukking van immuunreacties, de zogenaamde regulerende B (Breg) cellen [13, 23]. Breg-cellen reguleren immuunresponsen met name door het uitscheiden van de anti-inflammatoire cytokine IL-10 [24, 25]. Recente studies in autoimmuunziekten zoals systemische lupus erythematosis (SLE) [24] en reumatoïde artritis (RA) [26] hebben aangetoond dat de regulerende functie van Breg-cellen in deze aandoeningen verstoord is. Op grond van deze observaties werd in **hoofdstuk 5** de distributie van circulerende B-celpopulaties bepaald in patiënten met ANCA-geassocieerde kleine vaten vasculitis en vergeleken met de distributie zoals die aanwezig is in gezonde personen van dezelfde leeftijd en hetzelfde geslacht. Naast de frequenties van transitionele, naïeve en geheugen B-cellen werd ook de frequentie van CD19+CD24^{high}CD38^{high} en CD19+CD24^{high}CD27+ B-cellen bepaald. De twee laatstgenoemde B-celsubsets zijn in de literatuur beschreven als de Breg-populaties in de mens [24, 25]. Samengevat toonde deze studie aan dat de distributie van circulerende B-celpopulaties in patiënten met ANCA-geassocieerde kleine vaten vasculitiden afwijkt van de distributie in gezonde personen. Zo werd aangetoond dat het aantal circulerende CD19+CD24^{high}CD38^{high} B-cellen is afgenomen in patiënten met actieve ziekte ten opzichte van gezonde personen. Daarentegen waren CD19+CD24^{high}CD27+ B-cellen zowel tijdens actieve ziekte als in remissie verminderd. Aangezien productie van IL-10 gezien wordt als het belangrijkste kenmerk van Breg-cellen werd ook het percentage IL-10-producerende B-cellen bepaald, maar dit verschilde niet tussen patiënten en gezonde personen. Tot slot werden functionele studies uitgevoerd die lieten zien dat B-cellen van patiënten net zo goed in staat zijn om LPS-geïnduceerde TNF-α-productie door monocyten te onderdrukken als B-cellen van gezonde personen. Deze laatste studies werden alleen uitgevoerd met B-cellen van patiënten tijdens de rustige fase van de ziekte en daarom kan niet uitgesloten worden dat tijdens actieve ziekte de regulerende functie van B-cellen wel verstoord is. Een belangrijke vraag voor vervolgstudies is dan ook of een stabiele Breg-populatie van belang is om te voorkomen dat het ziekteproces weer actief wordt.

Daarnaast zijn er met betrekking tot het fenotype en de functie van Breg-cellen nog een aantal belangrijke vragen onbeantwoord. Zo kunnen IL-10-producerende

B-cellen gevonden worden in de transitionele, naïeve en geheugen B-celpopulaties. Dit suggereert dat het vermogen om IL-10 te produceren verworven kan worden tijdens verschillende stadia van de B-cel ontwikkeling [24, 27]. Ook wordt in de meeste studies een ligand voor TLR9 gebruikt om B-cellen IL-10 te laten produceren [24, 25, 27]. In dergelijke experimenten wordt IL-10-productie echter slechts in een beperkte populatie van B-cellen gevonden [28], terwijl bekend is dat alle B-cellen TLR9 tot expressie brengen. Dit suggereert dat er additionele mechanismen moeten zijn die bepalen of een B-cel wel of geen IL-10 kan produceren. Studies in CD4+ T cellen hebben aangetoond dat subsetspecifieke productie van cytokines epigenetisch bepaald wordt [29]. Er is bijvoorbeeld aangetoond dat de differentiatie van regulerende T-cellen (Treg) in Th17-cellen wordt gereguleerd door histone deacetylases (HDAC's) en dat remming van HDAC's dit proces onderdrukt [30]. Naar aanleiding van de bevindingen in T-cellen is in **hoofdstuk 6** onderzocht of de productie van pro- en anti-inflammatoire cytokines in primaire humane B-cellen ook afhankelijk is van HDAC's.

In deze experimenten werd het effect onderzocht van de HDAC-remmer Trichostatine A (TSA) op de cytokineproductie door (gestimuleerde) humane B-cellen. De resultaten van deze studies lieten zien dat TSA in B-cellen enerzijds de productie van IL-10, IL-6 en TGF-β remt en anderzijds de productie van TNF-α stimuleert. Verder werd aangetoond dat TSA de acetylatie van histon 3 (H3) en histon 4 (H4) in de promotorgebieden van de IL-10, TNF-α, IL-6 en TGF-β genen bevordert. Acetylatie van H3 en H4 wordt beschouwd als een epigenetisch proces dat geassocieerd is met actieve gentranscriptie. Derhalve kunnen deze resultaten dus niet verklaren waarom de expressie van IL-10, IL-6 en TGF-β geremd is door TSA. Het is bekend dat histonacetylases ook niet-histoneiwitten acetyleren en mogelijk heeft de acetylatie van deze niet-histoneiwitten een modulerend effect op de intracellulaire signaleringswegen die betrokken zijn bij de productie van cytokines door B-cellen. Een belangrijke transcriptiefactor voor de productie van IL-6 en TNF-α is Nuclear Factor-κB (NF-κB) en het is bekend dat acetylatie een modulerend effect heeft op de activiteit van deze factor. Additionele studies in **hoofdstuk 6** lieten zien dat een remmer van IKK-β, een factor die nodig is voor de klassieke route van NF-κB-activatie, het inhiberende effect van TSA op IL-10 en IL-6 productie van B-cellen verder versterkte. Een andere remmer (Bay 11-7082) die zowel de klassieke als de niet-klassieke route van NF-κB-activatie blokkeert, had echter een gedeeltelijk remmend effect op de TSA-geinduceerde TNF-α-productie. Samengevat tonen deze resultaten aan dat HDAC's een belangrijke rol spelen in de regulatie van cytokineproductie door B-cellen, maar dat de betrokken signaleringswegen zeer complex zijn. Verder onderzoek is noodzakelijk om deze signaleringswegen exact in kaart te brengen.

De studies beschreven in dit proefschrift dragen bij aan een beter begrip van de antistofafhankelijke en antistofonafhankelijke functies van B-cellen in ANCA-geassocieerde kleine vaten vasculitiden. Er is gevonden dat endogene factoren zoals BAFF en IL-21 de ANCA productie in een *in vitro* kweeksysteem bevorderen en dat dit effect nog verder kan worden versterkt wanneer tegelijkertijd TLR9 wordt geactiveerd. Verder is aangetoond dat de distributie van verschillende circulerende

B-celpopulaties in patiënten afwijkt van de distributie in gezonde personen. Het vermogen van de B-cellen om IL-10 te produceren en monocyten te onderdrukken is echter niet verstoord in patiënten. Deze studies geven aan dat het bestuderen van B-cellen op basis van alleen fenotypische merkers niet voldoende is om functionele effecten op de immuunrespons te kunnen bepalen. Vervolgstudies zijn nodig om de functies van B-cellen beter te begrijpen, bijvoorbeeld door het nauwkeuriger in kaart brengen van de cytokines die geproduceerd kunnen worden en door te bepalen onder welke omstandigheden dit plaatsvindt. In dit proefschrift is ook aangetoond dat HDAC's een rol spelen in de regulatie van cytokineproductie door B-cellen. Echter, aanvullende studies zijn noodzakelijk om te bepalen of remming van HDAC's een goede manier is om in B-cellen de productie van pro- en anti-inflammatoire cytokines te moduleren. Ten slotte impliceren de studies zoals beschreven in dit proefschrift dat een gedetailleerd kennis van B-cellen in ANCA-geassocieerde kleine vaten vasculitis zou kunnen bijdragen aan een beter inzicht in het ziekteverloop en wellicht aanknopingspunten biedt voor nieuwe behandelmogelijkheden.

REFERENCES

1. Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, Flores-Suarez LF, Gross WL, Guillevin L, Hagen EC, Hoffman GS, Jayne DR, Kallenberg CG, Lamprecht P, Langford CA, Luqmani RA, Mahr AD, Matteson EL, Merkel PA, Ozen S, Pusey CD, Rasmussen N, Rees AJ, Scott DG, Specks U, Stone JH, Takahashi K, Watts RA: **2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides.** *Arthritis Rheum* 2013, **65**(1):1-11.
2. Jennette JC, Falk RJ, Hu P, Xiao H: **Pathogenesis of antineutrophil cytoplasmic autoantibody-associated small-vessel vasculitis.** *Annu Rev Pathol* 2013, **8**:139-160.
3. Kallenberg CG, Stegeman CA, Abdulahad WH, Heeringa P: **Pathogenesis of ANCA-associated vasculitis: new possibilities for intervention.** *Am J Kidney Dis* 2013, **62**(6):1176-1187.
4. Hu N, Westra J, Kallenberg CG: **Dysregulated neutrophil--endothelial interaction in antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides: implications for pathogenesis and disease intervention.** *Autoimmun Rev* 2011, **10**(9):536-543.
5. Mulder AH, Heeringa P, Brouwer E, Limburg PC, Kallenberg CG: **Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA): a Fc gamma RII-dependent process.** *Clin Exp Immunol* 1994, **98**(2):270-278.
6. Falk RJ, Terrell RS, Charles LA, Jennette JC: **Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro.** *Proc Natl Acad Sci U S A* 1990, **87**(11):4115-4119.
7. Kettritz R, Jennette JC, Falk RJ: **Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils.** *J Am Soc Nephrol* 1997, **8**(3):386-394.
8. Chen M, Kallenberg CG: **The environment, geoepidemiology and ANCA-associated vasculitides.** *Autoimmun Rev* 2010, **9**(5):A293-8.
9. Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, Baslund B, Brenchley P, Bruchfeld A, Chaudhry AN, Cohen Tervaert JW, Deloukas P, Feighery C, Gross WL, Guillevin L, Gunnarsson I, Harper L, Hruskova Z, Little MA, Martorana D, Neumann T, Ohlsson S, Padmanabhan S, Pusey CD, Salama AD, Sanders JS, Savage CO, Segelmark M, Stegeman CA, Tesar V, Vaglio A, Wieczorek S, Wilde B, Zwerina J, Rees AJ, Clayton DG, Smith KG: **Genetically distinct subsets within ANCA-Associated Vasculitis.** *N Engl J Med* 2012, **367**(3):214-223.
10. Lepse N, Abdulahad WH, Kallenberg CG, Heeringa P: **Immune regulatory mechanisms in ANCA-associated vasculitides.** *Autoimmun Rev* 2011, **11**(2):77-83.
11. Stone JH, Merkel PA, Spiera R, Seo P, Langford CA, Hoffman GS, Kallenberg CG, St Clair EW, Turkiewicz A, Tchao NK, Webber L, Ding L, Sejismundo LP, Mieras K, Weitzenkamp D, Ikle D, Seyfert-Margolis V, Mueller M, Brunetta P, Allen NB, Fervenza FC, Geetha D, Keogh KA, Kissin EY, Monach PA, Peikert T, Stegeman C, Ytterberg SR, Specks U, RAVE-ITN Research Group: **Rituximab versus cyclophosphamide for ANCA-associated vasculitis.** *N Engl J Med* 2010, **363**(3):221-232.
12. Jones RB, Tervaert JW, Hauser T, Luqmani R, Morgan MD, Peh CA, Savage CO, Segelmark M, Tesar V, van Paassen P, Walsh D, Walsh M, Westman K, Jayne DR, European Vasculitis Study Group: **Rituximab versus cyclophosphamide in ANCA-associated renal vasculitis.** *N Engl J Med* 2010, **363**(3):211-220.
13. Lund FE, Randall TD: **Effector and regulatory B cells: modulators of CD4(+) T cell immunity.** *Nat Rev Immunol* 2010, **10**(4):236-247.
14. Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, Maeda N, Falk RJ, Jennette JC: **Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice.** *J Clin Invest* 2002, **110**(7):955-963.
15. Little MA, Al-Ani B, Ren S, Al-Nuaimi H, Leite M,Jr, Alpers CE, Savage CO, Duffield JS: **Anti-proteinase 3 anti-neutrophil cytoplasm autoantibodies recapitulate systemic vasculitis in mice with a humanized immune system.** *PLoS One* 2012, **7**(1):e28626.
16. Brouwer E, Tervaert JW, Horst G, Huitema MG, van der Giessen M, Limburg PC, Kallenberg CG: **Predominance of IgG1 and IgG4 subclasses of anti-neutrophil cytoplasmic autoantibodies (ANCA) in patients with Wegener's granulomatosis**

and clinically related disorders. *Clin Exp Immunol* 1991, **83**(3):379-386.

17. Brouwer E, Stegeman CA, Huitema MG, Limburg PC, Kallenberg CG: **T cell reactivity to proteinase 3 and myeloperoxidase in patients with Wegener's granulomatosis (WG).** *Clin Exp Immunol* 1994, **98**(3):448-453.
18. Pepper M, Jenkins MK: **Origins of CD4(+) effector and central memory T cells.** *Nat Immunol* 2011, **12**(6):467-471.
19. Crotty S: **Follicular helper CD4 T cells (TFH).** *Annu Rev Immunol* 2011, **29**:621-663.
20. Tangye SG, Ma CS, Brink R, Deenick EK: **The good, the bad and the ugly - TFH cells in human health and disease.** *Nat Rev Immunol* 2013, **13**(6):412-426.
21. Ettinger R, Sims GP, Robbins R, Withers D, Fischer RT, Grammer AC, Kuchen S, Lipsky PE: **IL-21 and BAFF/BLyS synergize in stimulating plasma cell differentiation from a unique population of human splenic memory B cells.** *J Immunol* 2007, **178**(5):2872-2882.
22. Ettinger R, Sims GP, Fairhurst AM, Robbins R, da Silva YS, Spolski R, Leonard WJ, Lipsky PE: **IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells.** *J Immunol* 2005, **175**(12):7867-7879.
23. Mauri C: **Regulation of immunity and autoimmunity by B cells.** *Curr Opin Immunol* 2010, **22**(6):761-767.
24. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, Mauri C: **CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients.** *Immunity* 2010, **32**(1):129-140.
25. Iwata Y, Matsushita T, Horikawa M, Dilillo DJ, Yanaba K, Venturi GM, Szabolcs PM, Bernstein SH, Magro CM, Williams AD, Hall RP, St Clair EW, Tedder TF: **Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells.** *Blood* 2011, **117**(2):530-541.
26. Flores-Borja F, Bosma A, Ng D, Reddy V, Ehrenstein MR, Isenberg DA, Mauri C: **CD19+CD24hiCD38hi B cells maintain regulatory T cells while limiting TH1 and TH17 differentiation.** *Sci Transl Med* 2013, **5**(173):173ra23.
27. van de Veen W, Stanic B, Yaman G, Wawrzyniak M, Sollner S, Akdis DG, Ruckert B, Akdis CA, Akdis M: **IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses.** *J Allergy Clin Immunol* 2013, **131**(4):1204-1212.
28. Tadema H, Abdulahad WH, Stegeman CA, Kallenberg CG, Heeringa P: **Increased expression of Toll-like receptors by monocytes and natural killer cells in ANCA-associated vasculitis.** *PLoS One* 2011, **6**(9):e24315.
29. Wilson CB, Rowell E, Sekimata M: **Epigenetic control of T-helper-cell differentiation.** *Nat Rev Immunol* 2009, **9**(2):91-105.
30. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I: **Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells.** *Blood* 2008, **112**(6):2340-2352.

Appendices

CONTRIBUTING AUTHORS

Abdulahad, Wayel H.[1]

Bijma, Theo[1]

Doornbos-van der Meer, Berber[1]

Gjaltema, Rutger A.F.[2]

Heeringa, Peter[2]

Huitema, Minke G.[1]

Kallenberg, Cees G.M.[1]

Land, Judith[1]

Lepse, Nikola[2]

Limburg, Pieter C.[3]

Rutgers, Abraham[1]

Stegeman, Coen A.[4]

Tadema, Henko[1]

[1]Department of Rheumatology and Clinical Immunology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

[2]Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

[3]Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

[4]Division of Nephrology, Department of Internal Medicine, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

ACKNOWLEDGEMENTS

During the past three and half years many people have contributed to the research that is presented in this thesis. Here I would like to express my appreciation for their support.

Firstly, I would like to acknowledge my promotores Prof. dr. Peter Heeringa, Prof. dr. Coen Stegeman and co-promotor Dr. Wayel Abdulahad under whose supervision I have worked during my PhD project. I got to know all of you already during the first research internship of my master's studies. While being part of ANCA research group as a student, I got the strong impression that you all worked well together as a team. After three and half years as your PhD student this impression has not changed.

Peter, it has been great to have you as my first promotor. Amongst the many things that I highly value in your working style, your "open door" approach is probably the one I have taken the greatest advantage of. It is difficult to remember a situation when you would not find time to discuss a piece of data or research-related issues. Probably, you will hear much less knocking on your door as I move away. Coen, I would like to thank you for all the contribution from you as a clinician. Particularly in the beginning of the project your contribution was indispensible and the research presented in this thesis would not be possible without your input. Wayel, one thing that has not changed over the years is your enthusiasm and passion for research. I am grateful to have you as my co-promoter. Your help with the experimental work especially in the beginning of the PhD project has with no doubt saved me a lot of time and effort. I truly admire and respect your devotion to the scientific research!

I also would like to sincerely thank Prof. dr. Cees Kallenberg and Dr. Bram Rutgers for their involvement in my PhD project. Cees, I thank you for your contribution to the clinical aspects of this work and for your involvement in the supervision of this project. Bram, I would like to express my gratitude for your input to this research work during our weekly B cell meetings and thanks for your time and effort to analyze a remarkable amount of clinical data!

I would like to acknowledge the members of the reading committee Prof. dr. N. Bos, Prof. dr. R. Goldschmeding and Prof. A. Salama for reading and approving my thesis.

Although I have been officially appointed in the department of Pathology and Medical Biology, most of my research time I have spent in the laboratory of Rheumatology and Clinical Immunology. I would like to thank Johan, Minke, Hannie, Gerda, Berber and Kiki for their support through the years. Theo, a special thanks for your help with chapter 6. It has been great to have such a driven and motivated collaborator like you! It's a pity we started our common work so late. It would be great if we could bring this project to a publishable end! I also thank the staff members from Medical Immunology Carolien, Jetske, Grytsje and Marieke for their help with ANCA-related work. Marcel, thanks for sharing your travelling stories, they always made me want to stop everything I do and go travel the world instead! ☺

I would like to thank the former and current PhD students from the Rheumatology and Clinical Immunology, Alexandre, Bert, Birgit, Deena, Fiona, Fleur, Henko, Judith, Koen, Lucas, Nan, Nishat, Nynke, Paulina, Qi, Sarah, Sebastian and Suzanne for creating a nice research environment in the lab. I wish you success with your research projects or other careers paths you have chosen! I also would like to acknowledge my former students Peter and Katinka for their contribution to their respective research projects.

Furthermore, I would like to thank the people from Transplantation Immunology, especially Joan, Niels, Magdalena and Richard, for the countless times they agreed to puncture my blood donors!

I would like to thank Geert, Henk and Roelof Jan from the FACS facility for all the help with cell sorting, which was always *gezellig*! Many thanks also to the technicians from MB-U lab, Jelleke, Henk Moorlag, Rianne and Peter Zwiers, for all the technical advice and support!

Thanks to my (former) colleagues from Medical Biology, Betty, Elise, Piotr, Niek, Gopi, Neng, Gesiena, Rebecca, Bram, Diane, Jelena, Jurjen, Nynke, Mojtaba, Mirjam and Sander; Eelke, Floor, Genaro, Leonie, Marlies and Sandra; Monika, Ewa, Ghazaleh, Ee Soo, Marloes, Ieneke and Jan Zijlstra. I also would like to thank Annet Bouman, Susan Pals and Hans van der Plas for their availability and assistance with administrative issues.

Rutger, thank you for your help with the epigenetic aspects of the chapter 6! I am sure that some of the experiments would not have gone so fast without your input!

Fahimeh, thank you for being such a great friend! I miss our common dinners and weekly coffee drinking in the city. I wish you the best of luck in Australia and I hope I can visit you there someday. David, thank you for your friendship and loyalty, you were a true friend when I needed one the most. Your laughter is literally unstoppable by the walls of UMCG! ;-)

Special thanks to my (former) office mates. Mirjan, it's been great to share the office with you, I enjoyed our ANCA-related and ANCA-unrelated discussions very much! Nato and Kasia, thank you for your friendship, and the immunological and not so immunological conversations we had. Kasia, I wish you all the best with your medical studies and future medical career. Nato, thanks for always finding time to listen! I wish you a lot of strength with the final steps of your PhD! Jill, it was great that you joined our office. I will miss those conversations when you suddenly start talking with Scottish accent and at the end say: "*You know what I mean*?" I always just nodded my head, but honestly, I have no clue what you mean when you speak with your lovely Scottish accent! ☺ Roel, I wish you the best of luck with your PhD! Despite several unexpected twists that you have experienced with your research projects, with no doubt you will complete your PhD successfully!

Many thanks to the climbing team – Kristiāna, Antoon, Ale-Watze, Koen, Niels, Qi and Pedro! I really miss the climbing evenings! I hope you can continue to master the tough art of climbing in the autumn when the boulder gym re-opens in a new place! Kristiān,

paldies par Tavu draudzību šo gadu garumā! Es vēlu Tev daudz izturības un veiksmes doktorantūrā! Es arī ļoti ceru, ka Tu mani apciemosi manā nākamajā mītnes zemē. ☺
Antoon and Ale-Watze, thank you for getting me acquainted with the amazing world of engineering! I would not have thought that the research in the field of engineering often works in a similar fashion as in the life sciences (i.e., trial & error)! After getting to know you guys, I am very suspicious whenever I meet an engineer! ;-)

My utmost thanks to my paranymphs! Dear Nato and Niels, I am very proud to have you by my side in my promotion! Nato, I have known you since I came to Groningen in 2008. We did the master studies together and afterwards at the same time started our PhDs in the same department. I have been lucky to have a friend and colleague like you! I am very happy that you have agreed to be my paranymph! My dear Niels, words do not seem to be enough to express my sincere gratitude for your love, friendship, support and patience! Thank you for having so much faith in me and for the continuous encouragement, which made the final stages of my PhD much easier. It is hard to imagine how I would have made it to this point without you!

Finally, I would like to thank my family. Visbeidzot, es vēlos pateikties savai ģimenei. Es esmu pavadījusi ārpus Latvijas gandrīz sešus gadus, bet šo gadu laikā es neesmu jutusies vientuļa, jo zinu, ka jūs man esat! Es esmu ļoti pateicīga saviem vecākiem par to, ka viņi vienmēr mani ir iedrošinājuši, sapratuši un atbalstījuši manus centienus izglītībā pat tad, kad nolēmu doties ārpus Latvijas. Mamm, paldies par nebeidzamiem uzmundrinājumiem un sapratni! Šī disertācija lielā mērā ir veltīta Tev! Savās domās esmu kopā ar jums visiem katru dienu!

Nikola